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Filed on 27 March 1998 (27.03.98)

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(54) Title: VEGF AND VEGF-C AS INFANT FORMULA SUPPLEMENTS

(57) Abstract

A composition, comprising an amount of VEGF-C sufficient to stimulate lymphatic angiogenesis in an intestine is provided. A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of the VEGF-C-containing composition sufficient to cause lymphatic angiogenesis in the infant's intestine is provided. A composition, comprising an amount of VEGF sufficient to stimulate vascular angiogenesis in an intestine is provided. A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of the VEFG-containing composition sufficient to cause vascular angiogenesis in the infant's intestine is also provided.

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A composition, comprising an amount of VEGF-C sufficient to stimulate lymphatic angiogenesis in an intestine is provided. A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of the VEGF-C-containing composition sufficient to cause lymphatic angiogenesis in the infant's intestine is provided. A composition, comprising an amount of VEGF sufficient to stimulate vascular angiogenesis in an intestine is provided. A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of the VEFG-containing composition sufficient to cause vascular angiogenesis in the infant's intestine is also provided.

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VEGF AND VEGF-C AS INFANT FORMULA SUPPLEMENTS AND RELATED METHODS

BACKGROUND OF THE INVENTION

The neonatal gut has a particular developmental vulnerability to necrotizing enterocolitis (NEC), an overwhelming infectious disease of neonatal intestine which is thought to have an ischemic vascular etiology (37). This disease is seen primarily in preterm, low birth weight infants, but may also occur in term newborns, particularly those exposed to the vasoconstrictive effects of cocaine. NEC causes significant morbidity (50%) and mortality (26%) and occurs endemically as well as epidemically. Necrosis and perforation are lowest in the areas of the gut which normally have the best perfusion (19,38). Because NEC is a disease limited to the neonatal period, there may be an acute precipitating event of vascular ischemia on top of a developmental component of relative vascular insufficiency.

There is experimental evidence for relative vascular insufficiency in the neonatal intestine. After total occlusion of a distal branch of the superior mesenteric artery, blood flow was reduced by 25% in 1 month old piglets compared with 75% in 1 day old piglets, and perfusion via collaterals from adjacent segments of bowel was significantly lower in the neonatal piglets (39). The etiology of this decreased collateral blood flow not been elucidated. Increased vascular resistance to flow or decreased blood vessel density in the neonatal intestine compared to the adult may predispose neonates to intestinal ischemic events, particularly in the ileo-cecal watershed area, which may lead to infarction and secondary bacterial invasion.

Lymphatic vessels may also play a role in development of NEC. Arterial and lymphatic ligation in neonatal piglets resulted in the complete histologic picture of NEC with full thickness necrosis, hemorrhage, mucosal/submucosal disruption,

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inflammatory infiltrates and gas in the intestinal wall, or pneumatosis intestinalis (40) which is pathognemonic for NEC in human.. Venous ligation alone induced small hemorrhages, while arterial ligation alone induced NEC-like lesions without the intramural gas found in pneumatosis intestinalis. Also the very lowest weight neonatal piglets developed NEC lesions with lymphatic ligation alone.

Lipid digestion in the small intestine has also been implicated in the pathogenesis of mucosal injury in the newborn animal. The epithelial mucosa lining the intestine must provide a barrier to potentially harmful agents, but allow permeation by beneficial macromolecules. This selective permeability of the intestine changes over the first months of life, during which time the intestines are particularly sensitive to the intraluminal mileau (59). Mucosal permeability is significantly greater in jejunum and ileum of piglets less than 2 weeks old compared to 1 month old animals after perfusion of the intestinal lumen with oleic acid, a long chain fatty acid (58). This increased permeability is dependent on the dose or concentration of the fatty acid, with greater concentration resulting in greater permeability and mucosal injury on histologic examination.

After ischemia and reperfusion of small intestine, mucosal permeability increase significantly more in 1 day old compared to 30 day old piglets whose intestinal lumens were exposed to the lipid component of formula versus a salt solution (47, 60). The carbohydrate and protein components of formula did not show this effect.

There is evidence that breast milk-fed, preterm babies have a lower incidence of NEC than formula fed infants, and that breast milk protects against bacterial translocation in animal models (41,42,43). However, there is no definitive understanding as to what aspect of breast milk or breast feeding produces this effect.

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A recent report suggests that VEGF and VEGF-C may be synergistic in promoting angiogenesis in some cell culture systems, such as bovine endothelial cell cultures in vitro [5]. VEGF and VEGF-C each have a synergistic angiogenic response with other growth factors, including transforming growth factor beta (TGF-β) and basic fibroblast growth factor (bFGF) on the same cell types *in vitro* [6,7]. There is currently no demonstration of such synergism *in vivo* in an animal or human system. Such synergism would not be obvious from the cell culture data, since cells *in vitro* usually behave differently than cells in an organ *in vivo*, as has been documented for theses endothelial cells [8]. They may have different receptor expression patterns and loss of organ specific characteristics such as fenestrations and electrical resistance. Receptor expression is particularly important in an organ such as the intestine, since there must be transport receptors for both these growth factors on the lumen side of the mucosa which are equally functional, and receptors for VEGF and VEGF-C on the microvascular and lymphatic endothelial cells within the intestinal submucosa. Thus, the *in vivo* roles of VEGF and VEGF-C have not been established.

Enteral/topical VEGF or VEGF-C use has not been described. Furthermore, there is currently no information on the effects of VEGF or VEGF-C in breast milk on the neonatal intestine. Thus, the present invention's application of enteral recombinant VEGF or VEGF-C to promote new blood vessel or lymphatic development in intestines of premature infants is novel. Likewise, the prophylactic use of VEGF and/or VEGF-C to prevent necrotizing enterocolitis in preterm newborns, and its application in other neonatal diseases, especially isolated intestinal perforation, post-operative NEC and short-gut syndrome are unique.

SUMMARY OF THE INVENTION

We have described for the first time the presence of Vascular Endothelial Growth Factor (VEGF) in human breast milk. VEGF's presence in breast milk indicates

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SUMMARY OF THE INVENTION

We have described for the first time the presence of Vascular Endothelial Growth Factor (VEGF) in human breast milk. VEGF's presence in breast milk indicates

that it has an effect on neonatal intestinal development of blood vessels. Breast milk from the first week postpartum has very high concentrations of VEGF, at approximately 300 times adult serum levels. VEGF levels in human milk decrease to a plateau at 4 - 5 weeks postpartum age (50 times adult serum levels) and remain fairly constant for the next 12 weeks.

We have also discovered the presence of Vascular Endothelial Growth Factor C (VEGF-C) in human breast milk.

VEGF and VEGF-C's presence in breast milk suggests that they have an effect on neonatal intestinal development of blood vessels and lymphatics. This may be particularly important in premature babies who have very immature intestinal development and are prone to necrotizing enterocolitis (NEC), an infection in the gut which can be life- threatening. NEC has been induced experimentally by decreasing blood flow to the intestine, particularly in the presence of an enteral formula feeding, allowing secondary invasion of bacteria into the wall of the intestine. Lymphatic vessels play important roles in absorbing and transporting macromolecules, fats, and immune cells and antibodies from intestines to the circulation. The under-development and immaturity of these lymphatic vessels may affect drainage of these substances from the intestine and may predispose premature newborns to NEC.

The present application describes the administration of VEGF and VEGF-C enterally and in infant formula to promote new blood and lymphatic vessel formation and prevent NEC, and giving VEGF-C enterally and /or topically during and after intestinal surgery to patients who have had part of their intestines removed in order to promote growth and recovery of the remaining intestine.

There are many important uses for the present invention. Addition of VEGF and/or VEGF-C to enteral feeds (formula or breast milk which is low in VEGF-C) or in

that it has an effect on neonatal intestinal development of blood vessels. Breast milk from the first week postpartum has very high concentrations of VEGF, at approximately 300 times adult serum levels. VEGF levels in human milk decrease to a plateau at 4 - 5 weeks postpartum age (50 times adult serum levels) and remain fairly constant for the next 12 weeks.

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sterile water or glucose water (with or without protein or fat to stabilize VEGF and VEGF-C) in preterm babies can promote new blood and lymphatic vessel formation and intestinal maturation, and to help prevent necrotizing enterocolitis (NEC). Enteral administration of VEGF and/or VEGF-C to the entire intestine can aide in gut recovery in NEC, shock, or in newborns who have had intestinal surgery. Use of VEGF and/or VEGF-C orally nasogastically, orogastrically, transpylorically, or via the stoma in post-operative short-gut patients can promote intestinal growth and maturation. Enteral administration of VEGF and/or VEGF-C can help protect babies exposed to cocaine, intrauterine growth retarded newborns, and premature newborns who are at risk for NEC. Use of topical application of VEGF and/or VEGF-C during surgical laparotomy to marginally viable areas of intestine can promote growth and recovery from intestinal injury or surgery.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Western blot showing VEGF isoforms in term breast milk. Reduced $VEGF_{165}$ standards on the left represent 1, 5, and 10ng. $VEGF_{165}$ shows two bands at 22 and 18 kDa. 5 μl reduced whole, aqueous and fat fractions of term milk were electrophoresed in the sample lanes. Whole milk has the two 22 and 18 kDa bands, but also a band at 14.5 kDa, which corresponds to the 121 VEGF isoform, and a band at 24 kDa which corresponds to higher molecular weight isoforms with more heparin binding affinity. A 5 μl aqueous milk sample contains the same, but somewhat weaker bands. 10 μl of a 1:1 fat-sodium taurocholate dilution was electrophoresed in the last lane but is not detectable at this level with this exposure. Whole milk reduced and treated with SDS loading buffer shows consistently more VEGF than aqueous fractions by Western blot representing liberation of VEGF from disruption of lipid membranes.

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Figure 2 is a Western blot of human breast milk samples, showing VEGF-C in 1) Aqueous milk from 33 weeks gestation, 3 days post-partum 2) Whole milk from 28 week gestation, day 1, 3) Aqueous milk from 28 week gestation, day 1, 4) Whole milk from 28 week gestation, day 1, 5) Aqueous milk from 28 week gestation, day 1, 6) Whole milk from term gestation, day 3, 7) Aqueous milk from term gestation, day 3.

Figure 3 shows the mean concentration of VEGF121 & 165 in nanograms per microliter in term whole milk and in the aqueous and fat components in the first week postpartum as measured by ELISA. Sample sizes are noted above each time point. Concentrations are very high in the first three days postpartum, and gradually decline over the second half of the first week to approximately 25 ng/ml in the aqueous and whole milk. Approximately 30% of the VEGF present in breast milk associates with the fat component, and this percentage does not change significantly with increasing postpartum age in the first week or thereafter. In this preparation, the VEGF concentration in whole milk is nearly identical to the aqueous fraction. VEGF associated with the lipid phase of whole milk seems to be antigenically unavailable until bile sales are added, as they are in the fat preparation.

Figure 4 is a graph of the concentration of VEGF in breast milk.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

As used in the specification and in the claims, "a" can mean one or more, depending upon the context in which it is used.

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As used in the specification and in the claims, "a" can mean one or more, depending upon the context in which it is used.

A composition, comprising an amount of VEGF sufficient to stimulate vascular angiogenesis in an intestine is provided. The composition can also include VEGF-C. The composition can further comprise water or glucose water, a protease inhibitor, a binding protein (e.g., heparin), bovine proteins (e.g., whey, casein, etc.), soy proteins, medium or long chain triglycerides, vegetable oils, liposomes, or other components not found in human milk. For topical administration, the components included with VEGF can include saline, other salts and other pharmaceutically acceptable compositions as are known in the art. In a composition for either topical or enteral administration, the composition can also include other active components that might be delivered to a patient suffering from ischemia, sepsis or lack of blood flow or an unrelated condition for which delivery to the intestine is desired. Examples of such additional components are numerous and are well known in the art. Other such active components may be developed or discovered, and are included in the present composition.

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The VEGF or VEGF-C of the invention may be isolated from naturally occurring sources (e.g., breast milk) as described herein or using any of the well-known methods for purifying or isolating proteins. The VEGF or VEGF-C used in the invention can be produced recombinantly. They can be expressed in a fusion protein including other protein moieties or they can be conjugated to carrier molecule to enhance their longevity, to enhance their activity or to enhance their resistance to degradation in the body. See, for example, U.S. Patent Nos. 5,219739 and 5,194,596, hereby incorporated by reference. Fragments of VEGF and VEGF-C that exhibit angiogenic activity can be used in the present methods and compositions. The fragments can be produced routinely using recombinant techniques, peptide synthesis techniques or by direct chemical modification of the protein (e.g., cleavage). The angiogenic activity of the fragment can be established using one of the assays shown in the examples or by other known assays..

A method of stimulating intestinal maturation in a premature infant is provided, comprising administering to the infant an amount of a VEGF-containing composition of the invention sufficient to cause vascular angiogenesis in the infant's intestine. The composition can be a VEGF-containing composition or the composition can contain both VEGF and VEGF-C or a heterodimer compound containing a subunit of both VEGF and VEGF-C, or a subunit of other growth factors that are synergistic with VEGF or VEGF-C. The composition can further comprise water or glucose water, a protease inhibitor, a binding protein (e.g., heparin), bovine proteins (e.g., whey, casein, etc.), soy proteins, medium or long chain triglycerides, vegetable oils, liposomes, carriers or other components not found in human milk. The VEGF-containing composition can be administered enterally (oral or otherwise) or topically. Administration can be to the lumen of the intestine or to the serosa of the intestine via intraperitoneal administration (e.g., lavage or injection). In the methods of the invention, the intestine can be the small intestine or large intestine.

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A method of stimulating intestinal healing in response to trauma is provided, comprising topically administering to the intestine an amount of VEGF sufficient to cause vascular angiogenesis in the infant's intestine. The VEGF can be in a composition as described above. The method can include administration of VEGF-C. The VEGF-containing composition can be administered enterally (oral or otherwise) or topically. Administration can be to the lumen of the intestine or to the serosa of the intestine via intraperitoneal administration (e.g., lavage or injection). The intestine can be small intestine or large intestine.

A method of stimulating new vessel development in the intestine is provided, comprising administering to a subject an amount of a VEGF-containing composition of the invention sufficient to cause angiogenesis in the subject's intestine. The method will produce vascular angiogenesis and may also produce lymphatic angiogenesis in combination with other components (e.g., VEGF-C). The VEGF-containing composition can be administered enterally (oral or otherwise) or topically. Administration can be to the lumen of the intestine or to the serosa of the intestine via intraperitoneal administration (e.g., lavage or injection). The intestine can be small intestine or large intestine.

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A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of a VEGF-C-containing composition of the invention sufficient to cause lymphatic angiogenesis in the infant's intestine. The composition can contain VEGF-C or it can contain both VEGF-C and VEGF or a heterodimer compound containing a subunit of both VEGF and VEGF-C, or other growth factors that are synergistic with VEGF or VEGF-C. The composition can further comprise water or glucose water, a protease inhibitor, a binding protein (e.g., heparin), bovine proteins (e.g., whey, casein, etc.), soy proteins, medium or long chain triglycerides, vegetable oils, liposomes, carriers or other components not found in human milk. The VEGF-C-containing composition can be administered enterally (oral or otherwise) or topically. Administration can be to the lumen of the intestine or to the serosa of the intestine via intraperitoneal administration (e.g., lavage or injection). The intestine can be small intestine or large intestine.

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A method of stimulating intestinal maturation in a premature infant is provided, comprising topically administering to the infant's intestine an amount of VEGF-C sufficient to cause lymphatic angiogenesis in the infant's intestine. The VEGF-C can be administered with other components such as carriers. The method can also include administration of VEGF. The VEGF-C-containing composition can be administered enterally (oral or otherwise) or topically. Administration can be to the lumen of the intestine or to the serosa of the intestine via intraperitoneal administration (e.g., lavage or injection). The intestine can be small intestine or large intestine.

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A method of stimulating new vessel development in the intestine is provided, comprising administering to a subject an amount of a VEGF-C-containing composition of the invention sufficient to cause angiogenesis in the subject's intestine. The method will produce vascular angiogenesis and may also produce lymphatic angiogenesis in combination with other components (e.g., VEGF). The VEGF-C-containing composition can be administered enterally (oral or otherwise) or topically. Administration can be to the lumen of the intestine or to the serosa of the intestine via intraperitoneal administration (e.g., lavage or injection). The intestine can be small intestine or large intestine.

The present compositions can be used to treat adult, children, infant and newborn large and small intestines. For example, after intestinal surgery, VEGF- or VEGF-C-containing compositions can be administered enterally or topically to facilitate recovery of the intestine in the area of the surgical trauma. Furthermore, because of their angiogenic activity, VEGF and VEGF-C can be used to treat any pathology related to ischemia or restricted blood flow and in any instance where enteral angiogenesis (vascular or lymphatic or both) would be beneficial. Administration can be to the lumen of the intestine or to the serosa of the intestine via intraperitoneal administration (e.g., lavage or injection).

The amounts of VEGF and/or VEGF-C to be administered are amounts effective to reduce (completely or partially) or prevent ischemia, necrosis or other pathology of the intestine associated with lack of adequate blood supply. The amounts of VEGF and/or VEGF-C to be administered are alternatively understood to be amounts effective to cause angiogenesis (vascular or lymphatic or both). Angiogenesis or the reduction or prevention of the targeted pathology can also be routinely assessed by the

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artisan practicing the methods described herein. The parameters and means for detecting and measuring angiogenesis are well known as are the parameters and means for identifying and quantifying improvements in intestinal pathologies. Thus, the determination of an effective amount can be readily determined in a clinical setting based on the knowledge in the art and the examples provided herein. Examples of effective concentrations in enteral and topical formulations include a range of about 100 picograms to about 200 nanograms per milliliter, and should be effective at higher concentrations and may be effective at lower concentrations. At higher concentrations, there is a risk of developing hypotension so monitoring is advised.

The following examples are provided to illustrate certain preferred embodiments of the invention, and do not suggest that the invention should be limited to the what they describe. Other embodiments are within the routine reach of the skilled artisan given the teaching provided herein.

EXAMPLES

Enteral Administration of VEGF and VEGF-C to Promote Intestinal Angiogenesis and Lymphangiogenesis

We have identified VEGF and VEGF-C in human milk for the first time. The present Examples are directed to the effects of VEGF in breast milk and VEGF added to formula on umbilical endothelial cells, a standard assay for new blood vessel formation, and to the in vivo effects of enteral administration of VEGF and VEGF-C on intestinal blood and lymphatic vessels in an animal model.

VEGF In Human Milk.

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For this study, freshly pumped breast milk samples at varying postpartum ages were collected, centrifuged at 1000 x g for 20 minutes at 40°C to separate the cellular,

fat, and aqueous components. Human milk leukocytes, the majority of which are macrophages, were then incubated in serum-free media for 24 hours in the presence or absence of Concanavalin A (10 ug/ml); conditioned media were collected and concentrated. Conditioned media and milk samples were analyzed by SDS-PAGE and Western blot for VEGF using a chemiluminescent detection system. Fetal small intestinal cell (FHS-74) proliferation studies were performed using Cell-Titer 96 AQ Assay (Promega), a non-radioactive colorimetric plate assay. VEGF concentrations in milk samples were measured by Quantikine ELISA (R & D Systems), an antibody-linked, enzyme activated, colorimetric assay.

We found VEGF concentrations were highest in the first 3 days postpartum in all fractions of human milk, whole (95 + 21 ng/ml), aqueous (92 + 18 ng/ml), and fat (31 + 5). VEGF levels then declined to a plateau around the 4th to 5th week postpartum (aqueous 15 + 8 ng/ml).

VEGF levels in fat comprise between 20 - 40% of the total, and this proportion does not change with gestational or postpartum age. In aqueous milk from days 4 - 7 postpartum, VEGF concentrations did not change significantly between term and preterm milk in the 37 - 42 week, 36, 35, 34, 33, or 27 week gestation milk samples we analyzed. Term human milk macrophages from the same postpartum time period did not seem to contribute significantly to VEGF concentrations, secreting only 144 + 25 pg/ml/24 hours/10^6 cells, which is approximately the same concentration of cells as in breast milk. VEGF levels in breast milk are 150 times cord serum levels from term babies after normal spontaneous vaginal delivery (650 + 140 pg/ml).

Human breast milk contains several isoforms of VEGF, including the predominant secreted isoforms, $VEGF_{165}$ and $VEGF_{121}$, and forms which also associate with the cell surface or extracellular matrix, $VEGF_{145}$, $VEGF_{189}$ or $VEGF_{206}$. In Western blots of whole and aqueous milk samples, we demonstrated 4 predominant

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In summary, VEGF levels are very high in the first week postpartum in both term and preterm milk (approximately 500 times adult serum levels), and gradually declined to a plateau around the 4th to 5th week (70 times adult serum levels). An increase in proliferation of the intestinal epithelial cells with VEGF exposure was not shown. This data suggests that VEGF and VEGF-C are secreted into breast milk by mammary epithelial cells and may promote angiogenesis and lymphangiogenesis in the developing neonatal intestine.

VEGF-C In Human Milk.

We have also discovered the presence of Vascular Endothelial Growth Factor C (VEGF-C) in human breast milk. The protocol is as described above, except that the antibody used in the Western blot is specific for VEGF-C and is non-crossreactive with VEGF.

VEGF-C is related to VEGF, but regulates endothelial cells in lymphatic vessels. VEGF-C has been shown to stimulate lymphatic vessel formation in transgenic mice. VEGF and VEGF-C's presence in breast milk suggests that they have an effect on neonatal intestinal development of blood vessels and lymphatics. This may be particularly important in premature babies who have very immature intestinal development and are prone to necrotizing enterocolitis (NEC), an infection in the gut which can be life- threatening. NEC has been induced experimentally by decreasing blood flow to the intestine, particularly in the presence of an enteral formula feeding,

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allowing secondary invasion of bacteria into the wall of the intestine. Lymphatic vessels play important roles in absorbing and transporting macromolecules, fats, and immune cells and antibodies from intestines to the circulation. The under- development and immaturity of these lymphatic vessels may affect drainage of these substances from the intestine and may predispose premature newborns to NEC.

Purification of VEGF and VEGF-C isoforms.

VEGF and VEGF-C isoforms will be purified from human breast milk by sephadex chromatography followed by HPLC, and the mitogenic activity of different VEGF and VEGF-C isoforms will be assayed in human umbilical endothelial cells (HUVEC) in vitro.

Size exclusion chromatography: Sephadex chromatography will be performed using a commercially available sepharose. Approximately 60 cc of aqueous breast milk will be prepared by centrifugation at 100,000 x g at 4°C to separate the liposomes, lyophilized, and brought up in PBS, then incrementally applied over a sephadex column. The sepharose will be washed prior to elution with 0.1 M sodium citrate, 0.3 M sodium chloride elution buffer. The VEGF will be eluted with 0 to 1 M NaCl stepwise gradient and dialyzed against 20 volumes of PBS at 40°C as previously described (50).

HPLC: Protein eluted from sephadex, size exclusion chromatography will be loaded onto Vydac VHP anion exchange column (25 x 5mm) with a dual-pump system, pre-equilibrated in 0.01M Tris HCl, pH 6.5 according to established protocols (50,51). Elution will be performed at ambient temperature with a 0 to 0.25M NaCl linear gradient at a flow rate of 1 ml/min. Absorbance at 280 nm will be monitored by spectroscopy. All VEGF proteins will be measured by quantitative ELISA and evaluated qualitatively by western blot.

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Western blot: Sizes of VEGF and VEGF-C will be verified by Western blot using a polyclonal antibody which recognizes VEGF or VEGF-C, with no cross reactivity (Santa Cruz Biotechnology), followed by chemiluminescent detection.

Human breast milk: Human breast milk, approximately 50 - 60 L, has already been collected and stored in -20°C freezer for the study of growth factors under another research protocol.

We designed this study to test the hypothesis that VEGF and VEGF-C in breast milk promote angiogenesis and lymphangiogenesis in the neonatal intestine. We will use a neonatal piglet model, which has been studied extensively, and best approximates the intestinal mucosal conditions found in the human neonate (16,17,18,19).

Mitogenic Assay of human milk-derived VEGF activity in HUVEC.

HUVEC culture: Human umbilical vein epithelial cells, HUVEC (ATCC), are currently being grown in our laboratory with endothelial growth cell supplement and 20% FBS, 5 mg/ml heparin, 4 mM L-glutamine in F-12K media (Kaign's modification of Hamm's F12) on gelatin-coated plates.

HUVEC Proliferation Assay: Serial dilutions of human milk-derived VEGF and VEGF-C are tested by proliferation of human umbilical vascular endothelial cells in vitro, a standard assay for angiogenic activity (20,21). Briefly, HUVEC will be seeded at 5 x 104 cells per 0.5 ml per well on a 96 well, gelatin-coated plate, and cultured in the presence of serial dilutions of human milk-purified VEGF or VEGF-C (0.01 to 100 ng/ml) (52), a dilute concentration of breast milk (1:500), or assay media alone (F-12K, 10% FBS, 4 mM glutamine) for 72 hours. A colorimetric proliferation assay (Cell Titer 96, Promega) will allow quantification of cell numbers via an ELISA plate reader.

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Human milk-purified VEGF and VEGF-C will also be incubated with gastric fluids obtained from preterm human newborns for 1 hour at 37°C and activity verified by proliferation assay of HUVEC cells as described. Proteolysis will also be assessed by western blot.

Human milk VEGF proteins can have full mitogenic activity in HUVEC culture. VEGF-treated cells numbers will be 130% of control.

Alternative methods: Lipid components of breast milk could be carried over from sephadex chromatography and hamper HPLC purification. We have demonstrated that aqueous human milk may still have some intact liposomes and liposomal membranes after centrifugation at 2000 x g for 10 minutes. We spiked aqueous milk fraction with a fluorescent-tagged phosphotidylcholine, centrifuged at 100,000 x g for 16 hours at 4°C through a 5 to 30% sucrose gradient. Liposomes were noted fluorescing in the first two fractions, corresponding to a frothy white layer at the top. Fractions were then assayed for VEGF by ELISA. VEGF was not found associated with the small liposomes, but 60% of the VEGF was found predominantly in the purely aqueous fraction, at 12-15% sucrose. The cleared aqueous fraction should give good results with this purification scheme.

VEGF and VEGF-C in breast milk stimulate angiogenesis and lymphangiogenesis in the neonatal intestine.

VEGF and VEGF-C increase blood vessel and lymphatic vessels density in the neonatal piglet intestine by immunohistochemistry and microvessel counting in sections of intestine from piglets given formula alone, human-milk purified VEGF and VEGF-C with formula, and sow suckled piglets.

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Alternative methods: Lipid components of breast milk could be carried over from sephadex chromatography and hamper HPLC purification. We have demonstrated that aqueous human milk may still have some intact liposomes and liposomal membranes after centrifugation at 2000 x g for 10 minutes. We spiked aqueous milk fraction with a fluorescent-tagged phosphotidylcholine, centrifuged at 100,000 x g for 16 hours at 4°C through a 5 to 30% sucrose gradient. Liposomes were noted fluorescing in the first two fractions, corresponding to a frothy white layer at the top. Fractions were then assayed for VEGF by ELISA. VEGF was not found associated with the small liposomes, but 60% of the VEGF was found predominantly in the purely aqueous fraction, at 12-15% sucrose. The cleared aqueous fraction should give good results with this purification scheme.

VEGF and VEGF-C in breast milk stimulate angiogenesis and lymphangiogenesis in the neonatal intestine.

VEGF and VEGF-C increase blood vessel and lymphatic vessels density in the neonatal piglet intestine by immunohistochemistry and microvessel counting in sections of intestine from piglets given formula alone, human-milk purified VEGF and VEGF-C with formula, and sow suckled piglets.

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The pattern of VEGF secretion in human milk is highest when the gut is most immature and open to macromolecules, tapering off to a plateau around the 4th to the 5th week postpartum (11). For some period of time postnatally, VEGF and VEGF-C may be able to cross the mucosal epithelial cell barrier and stimulate angiogenesis and lymphangiogenesis. Thus, the target organ for VEGF proteins in breast milk is the newborn small intestine, possibly the ileocecal watershed area.

The neonatal piglet model is the closest non-primate animal model for studies of neonatal nutrition and ischemia-reperfusion injury, or experimental NEC (16,17,19,44,53). Human newborns have similar transit times, digestion, and morphology and physiology of the gastroinestinal system as neonatal piglets. Specifically, tissues of the lamina, muscularis, submucosa and serosa are virtually identical. Piglets grow at a faster rate, however, and a comparable period of development in the suckling piglet is birth to 6 weeks compared with birth to 6 months in the human infant. Neonatal piglets can also be reared on their mother's milk, human infant formula, or cow's milk, and the composition of human and swine milk are qualitatively similar (45).

The neonatal piglet model is the model which most closely resembles human neonatal NEC, has also been used extensively to study the effects of ischemia-reperfusion and cocaine on decreasing gut perfusion in the neonatal intestine (19,46). The model is particularly suitable since there is also a well documented age-related increase in vulnerability to mucosal injury induced by ischemia-reperfusion. The neonatal piglet has also been shown to be more vulnerable to this type of injury in the presence of formula compared with 1 month old piglets, similar to human newborns (47). Neonatal piglets, whose intestinal lumens were perfused with oleic acid to induce injury, showed greater increases in mucosal permeability compared with 1 month old piglets (48). In smaller birth weight piglets, the terminal ileum is less developed, and

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small for gestational age newborn piglets have shown to have spontaneous ischemic mucosal lesions in the terminal ileum, and rarely transmural necrosis (49).

Animal care and preparation: Pregnant Yucatan pigs in their 2nd to 5th pregnancy are used, and piglets of either sex will be separated from mother within 6 hours after birth in the VEGF-formula and formula alone groups, or allowed to have sow's milk for the suckling group (n= 3 each group). Piglets in the VEGF-formula and formula alone groups will be fed cow's milk based formula (Nutren 1.0, Clintec, Deerfield, IL) ad libitum every 2 hours for the first week, then every 3 - 4 hours in the second to third week. Human milk-purified VEGF and VEGF-C will be stored at -20°C and added to formula no more than 8 hours prior to use. The amount of VEGF and VEGF-C added to formula will be based on the amount in human milk for the corresponding post-partum day. VEGF-formula will be stored at 4°C. All formula will be warmed with running warm water for 10 minutes prior to feeding. Heat lamps will provide warmth for the piglets separated at birth.

Miniature Yucatan piglets are fairly hardy, thriving even when separated from mother at birth, and grow well on cow's milk formula. VEGF supplementation of formula at the same concentration and time course as we have shown in human milk should provide a representation of the effects of VEGF and VEGF-C on blood and lymphatic vessel development in the neonate. However, VEGF proteins may not act alone in promoting angiogenesis, and indeed, has been shown to act synergistically with bFGF in rabbits (55). Therefore, two control groups of piglets are indicated: one group which is also separated at birth and fed only formula, and the sow-suckled group which has the added benefit of all the possible angiogenic factors in breast milk.

Purification of VEGF from human breast milk: rhVEGF $_{165}$ has been used successfully to stimulate angiogenesis in a variety of animal models, including the pig in 10 days up to 30 days (56). rhVEGF $_{165}$ is soluble, but also binds well to heparin and

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Animal Surgery: At 14 days of age, piglets will be anesthetized with isofluorane, acepromazine 0.8 mg/kg and ketamine 50 mg/kg. The peritoneal cavity will be opened through a midline incision.

Angiography: The intestinal sections will be assessed by angiography using Omnipaque, a water soluble radio-opaque contrast media, 5 - 10 cc, after cannulating the superior mesenteric artery with a 3.5 French infusion catheter. The intestine will be divided into duodenum from the pylorus to the peritoneal reflection (i.e. ligament of Treitz), jejunum, the proximal half of the remaining small intestine, ileum, the distal half of small intestine, and proximal colon. To standardize the location of angiographic and block tissue sampling between variable lengths of intestine, the duodenal sample will be taken from just distal to the pylorus and proximal to the ampulla of Vater; the jejunal sample, from just beyond the ligament of Treitz; the ileal sample, just before the ileocecal valve; and the proximal colon sample, at the hepatic flexure. The catheter will be advanced to the take-off of the jejunal or ileal branch from the superior mesenteric artery and the intestinal loop will be laid out prior to injection to minimize overlapping sections. Scale markers will be positioned prior to injection by an automated angiographic injector (Medrad, Pittsburgh, PA) at a rate of 1 ml/sec. Serial

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Immunohistochemistry: The entire intestine will be excised and quickly placed in ice-cold saline then divided according to the guidelines above. Each intestinal segment will be weighed. The intestinal sections will be treated with formalin fixation and cryopreservation of two 2 cm blocks of tissue from each region. Block tissues will be sampled according to the format in the angiography section. Anti-human PECAM antibody (Dako Polyclonal, Santa Barbara, CA) or anti-vWf will be used for immunoperoxidase staining of vascular endothelial cells or anti-desmoplakin I and II, expressed in lymphatic endothelial cells. If neither human antibody cross reacts with these porcine endothelial cell markers, then alkaline phosphatase staining of endothelial cells will be performed using an indoxyl-tetrazolium method. Staining will be carried out for 1 hour at 37°C using nitroblue tetrazolium (30 mg) and 5-bromo 4-chloro 3-indolyl phosphate p-toluidine salt (6 mg), in buffer: 6.9 mM Mg_{SO4} and 27.5 mM $NaBO_2$, pH 9.2 to 9.4 (adjusted with boric acid). Slides will be rinsed and counterstained with eosin (57).

Microvessel Density: Microvessel counting to determine blood vessel density will be done per unit area, but also normalized to the entire circumferential section of intestine to counter any differences in intestinal weight, edema, or atrophy. Blood vessels will be identified by immunostaining of endothelial cell specific markers, PECAM or von Willebrand's factor (vWf), and counted. We will also obtain corroborative data by angiography, and vessel counting of set area of the radiographs. The most vascular area of the tissue section will be determined by scanning the slide with the low power objective. Using a 20X objective lens (for 200x magnification), three fields on each section will be counted at 0.6362 mm^2 per field per standard protocol for the mean

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number of vessels per mm^2. A vessel per Weidner's definition is "any endothelial cell of endothelial cell cluster which is clearly separate from other adjacent microvessels, and other connective tissue elements" is included in the count. "Vessel lumens, although usually present were not necessary for a structure to be defined as a microvessel, and red blood cells were not used to define a lumen ."(58) Differential immunochemical staining of vascular and lymphatic vessels will be used to differentiate the these elements.

Statistical Analysis: Mean microvessel counts for each intestinal segment in the VEGF-formula and formula alone groups will be analyzed using student t test. Differences in mean microvessel counts among VEGF-formula, formula alone, and sow-suckled groups will be analyzed by ANOVA. With a significance of 0.05 and 2-sided alpha, power of .80, formula mean number of blood vessels/ mm2 are estimated as 160 bv/mm^2 from rabbit ischemic-hind limb therapeutic response to VEGF, while VEGF-formula treated group's estimated mean is 235 bv/ mm^2 with a standard deviation of 35 (56). It is estimated we will need 3 cases in each group. Standard litter sizes are around 5 to 6 piglets, allowing for a 10% attrition rate in each group.

The VEGF-formula group should show significantly more microvessel vascular and lymphatic density than the formula alone group in all sections studied, with the most significant differences in the terminal ileum at the watershed area of the intestinal circulation. We also expect the sow-suckled group to exhibit synergistic effects of multiple angiogenic factors with the highest vessel count. Counts from angiography are expected to correlate with those from immunohistochemistry.

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Daily administration of VEGF and VEGF-C over 14 days may be too short to see maximal effect of enterally administered VEGF on microvessel development. However, single bolus injection of VEGF protein and VEGF cDNA produces significant increases in capillary density within 10-30 days in an ischemic animals and in humans (54,56). We have chosen 14 days as a more cost effective alternative to 1 month, while still ensuring adequate time for blood vessel formation to reach statistical significance.

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The control groups are not exactly equivalent since one group is allowed to stay with their sow, and the other two groups are removed from the mother. However, to isolate the effects of VEGF and VEGF-C, oral supplementation appears to be the best method compared with intermittent VEGF antibody administration.

Formulations of Infant Formula Containing VEGF and VEGF-C.

VEGF, either recombinant or human-milk purified is added to infant formula or given enterally in approximately the same concentrations as found in human breast milk. Approximately 95 nanograms per milliliter (ng/ml) in the first 3 days of therapy to 15 ng/ml from week 5 on as shown in graph Figure 4. Protease inhibitors may also be added to formula to help preserve VEGF and VEGF-C's bioactivities. VEGF-C will be added according to similar guidelines.

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Anti-human endothelial marker antibodies may not cross react with their cell antigens, as stated. If neither human antibody cross reacts with these porcine endothelial cell markers, then alkaline phosphatase staining of endothelial cells will be performed using an indoxyl-tetrazolium method, and counterstaining with eosin (57).

The control groups are not exactly equivalent since one group is allowed to stay with their sow, and the other two groups are removed from the mother. However, to isolate the effects of VEGF and VEGF-C, oral supplementation appears to be the best method compared with intermittent VEGF antibody administration.

Formulations of Infant Formula Containing VEGF and VEGF-C.

VEGF, either recombinant or human-milk purified is added to infant formula or given enterally in approximately the same concentrations as found in human breast milk. Approximately 95 nanograms per milliliter (ng/ml) in the first 3 days of therapy to 15 ng/ml from week 5 on as shown in graph Figure 4. Protease inhibitors may also be added to formula to help preserve VEGF and VEGF-C's bioactivities. VEGF-C will be added according to similar guidelines.

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VEGF and VEGF-C can be combined until the baby is given breast milk or until no further advantage of these growth factors has been demonstrated.

VEGF and VEGF-C can be diluted in water, saline or glucose containing solutions prior to being given enterally or added to formula. Protease inhibitors, similar to those present in breast milk, may also be added.

Breast milk of mothers of premature babies is assayed for VEGF and VEGF-C concentrations. If low (<15 ng/ml at anytime, or less than half the expected concentration in first 4 weeks post-partum) VEGF or VEGF-C may be added to the mother's milk to bring the concentration up to a normal level.

If the baby is very ill or has NEC, prolonged addition of VEGF and VEGF-C at high levels to breast milk, formula or enterally may be necessary to ensure gut recovery (high levels ~ 100 ng/ml).

Topical Administration of VEGF and VEGF-C to Intestine.

VEGF and/or VEGF-C as 15 to 100 ng/ml in water, normal saline or glucose containing solutions, is used to irrigate the intestinal lumen of the bowel during surgery. Marginally viable areas of bowel as well as surgical anastomoses after resection of non-viable bowel could receive this topical application.

In babies who are not candidates for surgery, irrigation of the peritoneum can be performed through a mini-laparotomy incision before drain placement, and on a daily basis after drain placement, to improve gut recovery. This irrigation is topical application to the serosa of bowel, not the intestinal lumen. It can be continued as long as the drain is in place.

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Post-operatively, enteral administration of VEGF and VEGF-C in the noted concentrations can be continued several times a day to improve gut recovery and to enhance gut growth in babies with short-gut syndrome.

Enteral administration in the noted formulations and concentrations can also be given to babies who have experienced shock, cocaine prenatally, or are intrauterine growth retarded and may have compromised blood and lymphatic flow to and from the intestines.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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What is claimed is:

1. A composition, comprising an amount of VEGF-C sufficient to stimulate lymphatic angiogenesis in an intestine.

2. A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of the composition of claim 1 sufficient to cause lymphatic angiogenesis in the infant's intestine.

3. The method of claim 2, wherein the VEGF-C is administered enterally.

4. The method of claim 2, wherein the intestine is small intestine.

5. A method of stimulating intestinal maturation in a premature infant, comprising topically administering to the infant's intestine an amount of VEGF-C sufficient to cause lymphatic angiogenesis in the infant's intestine.

6. The method of claim 5, wherein the intestine is small intestine.

7. A composition, comprising an amount of VEGF sufficient to stimulate vascular angiogenesis in an intestine.

8. A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of the composition of claim 7 sufficient to cause vascular angiogenesis in the infant's intestine.

9. The method of claim 8, wherein the composition is administered enterally.

10. The method of claim 8, wherein the intestine is small intestine.

What is claimed is:

1. A composition, comprising an amount of VEGF-C sufficient to stimulate lymphatic angiogenesis in an intestine.

2. A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of the composition of claim 1 sufficient to cause lymphatic angiogenesis in the infant's intestine.

3. The method of claim 2, wherein the VEGF-C is administered enterally.

4. The method of claim 2, wherein the intestine is small intestine.

5. A method of stimulating intestinal maturation in a premature infant, comprising topically administering to the infant's intestine an amount of VEGF-C sufficient to cause lymphatic angiogenesis in the infant's intestine.

6. The method of claim 5, wherein the intestine is small intestine.

7. A composition, comprising an amount of VEGF sufficient to stimulate vascular angiogenesis in an intestine.

8. A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of the composition of claim 7 sufficient to cause vascular angiogenesis in the infant's intestine.

9. The method of claim 8, wherein the composition is administered enterally.

10. The method of claim 8, wherein the intestine is small intestine.

What is claimed is:

1. A composition, comprising an amount of VEGF-C sufficient to stimulate lymphatic angiogenesis in an intestine.

2. A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of the composition of claim 1 sufficient to cause lymphatic angiogenesis in the infant's intestine.

3. The method of claim 2, wherein the VEGF-C is administered enterally.

4. The method of claim 2, wherein the intestine is small intestine.

5. A method of stimulating intestinal maturation in a premature infant, comprising topically administering to the infant's intestine an amount of VEGF-C sufficient to cause lymphatic angiogenesis in the infant's intestine.

6. The method of claim 5, wherein the intestine is small intestine.

7. A composition, comprising an amount of VEGF sufficient to stimulate vascular angiogenesis in an intestine.

8. A method of stimulating intestinal maturation in a premature infant, comprising administering to the infant an amount of the composition of claim 7 sufficient to cause vascular angiogenesis in the infant's intestine.

9. The method of claim 8, wherein the composition is administered enterally.

10. The method of claim 8, wherein the intestine is small intestine.

11. A method of stimulating intestinal maturation in a premature infant, comprising topically administering to the infant's intestine an amount of VEGF sufficient to cause vascular angiogenesis in the infant's intestine.

12. The method of claim 11, wherein the intestine is small intestine.

11. A method of stimulating intestinal maturation in a premature infant, comprising topically administering to the infant's intestine an amount of VEGF sufficient to cause vascular angiogenesis in the infant's intestine.

12. The method of claim 11, wherein the intestine is small intestine.

11. A method of stimulating intestinal maturation in a premature infant, comprising topically administering to the infant's intestine an amount of VEGF sufficient to cause vascular angiogenesis in the infant's intestine.

12. The method of claim 11, wherein the intestine is small intestine.

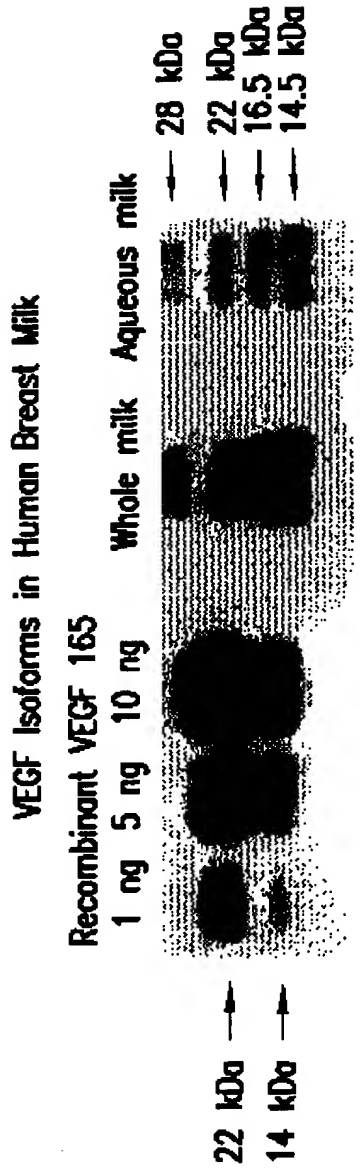

VEGF Isoforms in Human Breast Milk
Recombinant VEGF 165
1 ng 5 ng 10 ng Whole milk Aqueous milk
28 kDa
22 kDa
16.5 kDa
14.5 kDa
22 kDa
14 kDa

FIG. 1

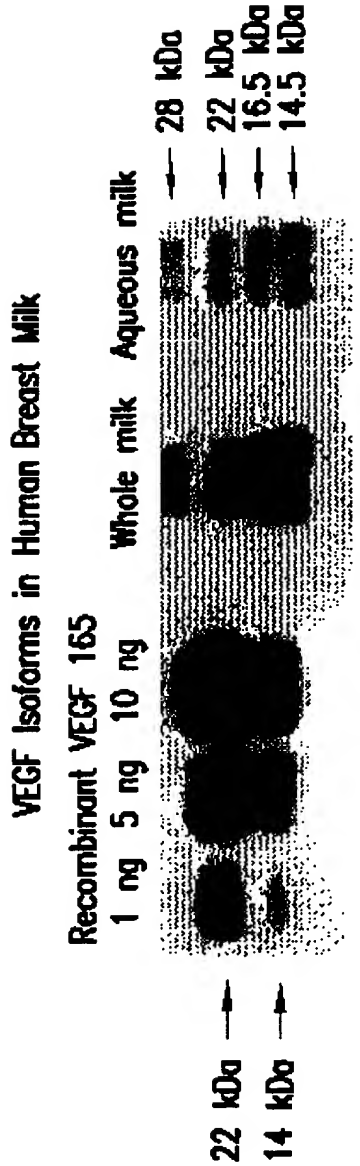

VEGF Isoforms in Human Breast Milk
Recombinant VEGF 165
1 ng 5 ng 10 ng
Whole milk
Aqueous milk
28 kDa
22 kDa
16.5 kDa
14.5 kDa
22 kDa
14 kDa

FIG. 1

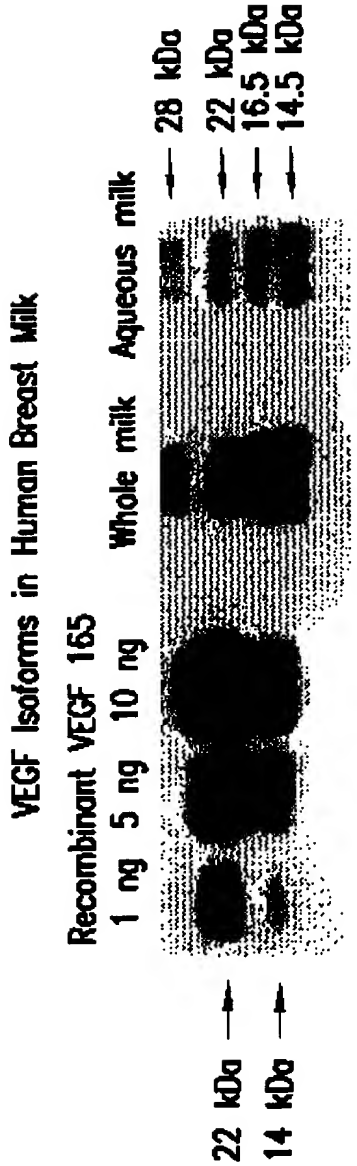
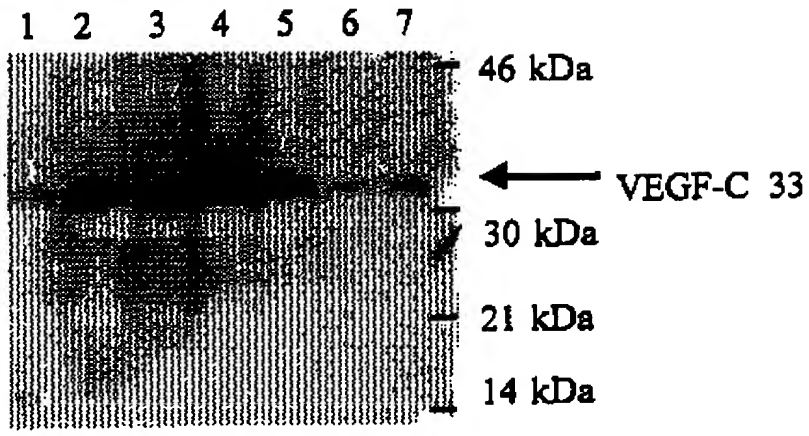

VEGF Isoforms in Human Breast Milk
Recombinant VEGF 165
1 ng 5 ng 10 ng
Whole milk
Aqueous milk
28 kDa
22 kDa
16.5 kDa
14.5 kDa
22 kDa
14 kDa

FIG. 1

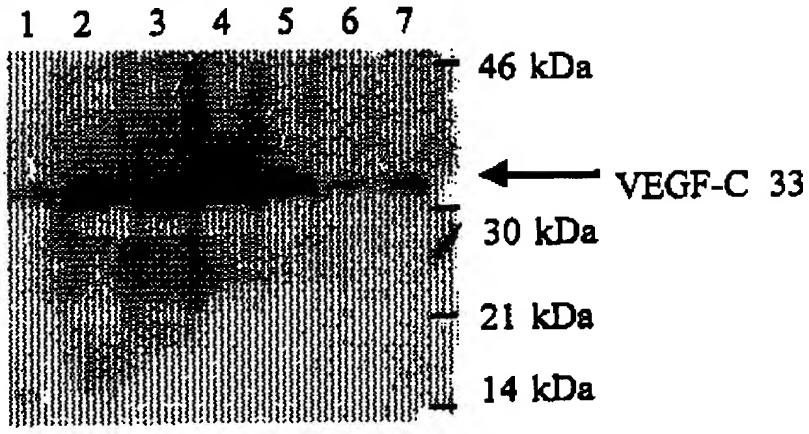
Human Breast Milk Samples



1. Aqueous milk from 33 weeks gestation, 3 days post-partum
2. Whole milk from 28 weeks gestation, day 1
3. Aqueous milk from 28 weeks gestation, day 1
4. Whole milk from 28 weeks gestation, day 1
5. Aqueous milk from 28 weeks gestation, day 1
6. Whole milk from term gestation, day 3
7. Aqueous milk from term gestation, day 3

FIG.2

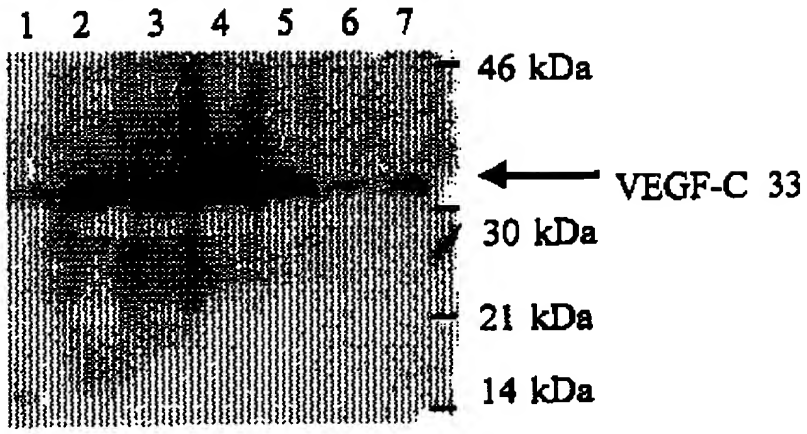
Human Breast Milk Samples



1. Aqueous milk from 33 weeks gestation, 3 days post-partum
2. Whole milk from 28 weeks gestation, day 1
3. Aqueous milk from 28 weeks gestation, day 1
4. Whole milk from 28 weeks gestation, day 1
5. Aqueous milk from 28 weeks gestation, day 1
6. Whole milk from term gestation, day 3
7. Aqueous milk from term gestation, day 3

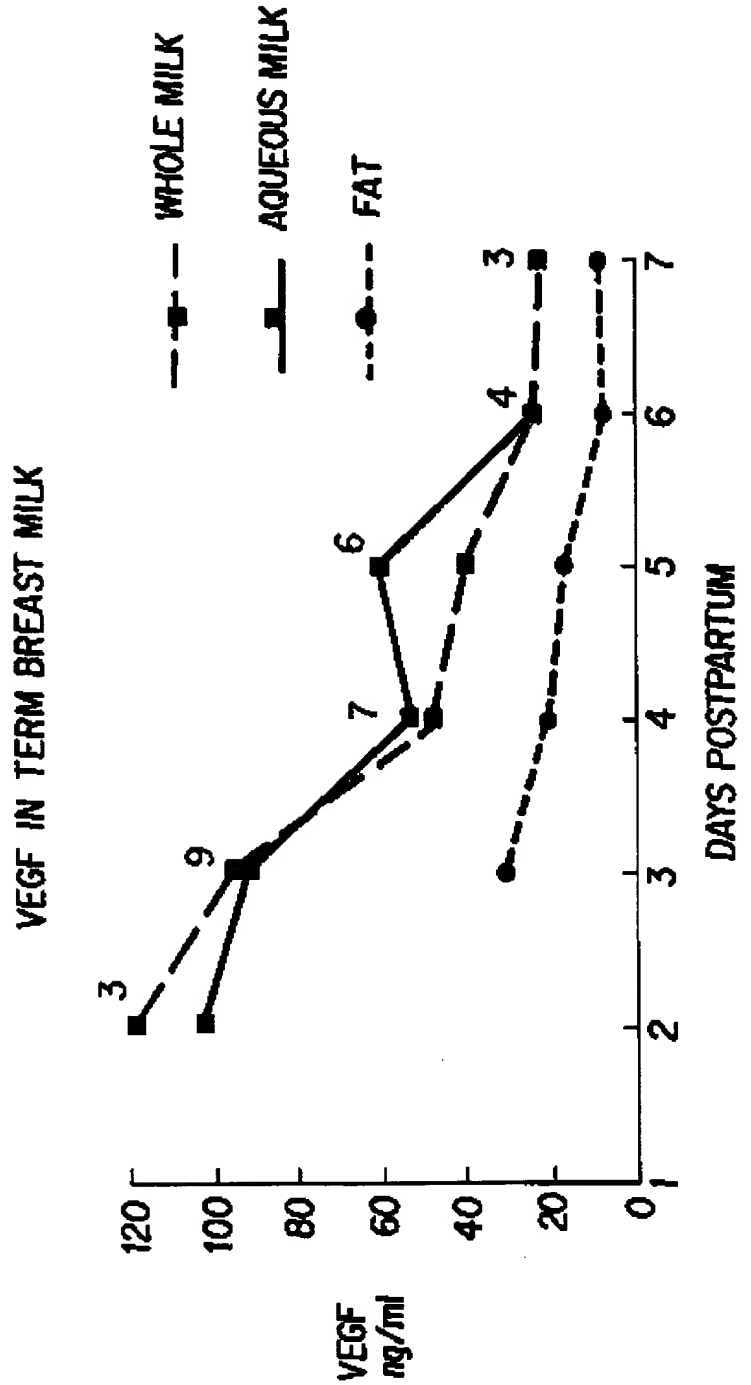
FIG.2

Human Breast Milk Samples



1. Aqueous milk from 33 weeks gestation, 3 days post-partum
2. Whole milk from 28 weeks gestation, day 1
3. Aqueous milk from 28 weeks gestation, day 1
4. Whole milk from 28 weeks gestation, day 1
5. Aqueous milk from 28 weeks gestation, day 1
6. Whole milk from term gestation, day 3
7. Aqueous milk from term gestation, day 3

FIG.2


VEGF IN TERM BREAST MILK
WHOLE MILK
AQUEOUS MILK
FAT
VEGF mg/ml
120
100
80
60
40
20
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1
2
3
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7
DAYS POSTPARTUM
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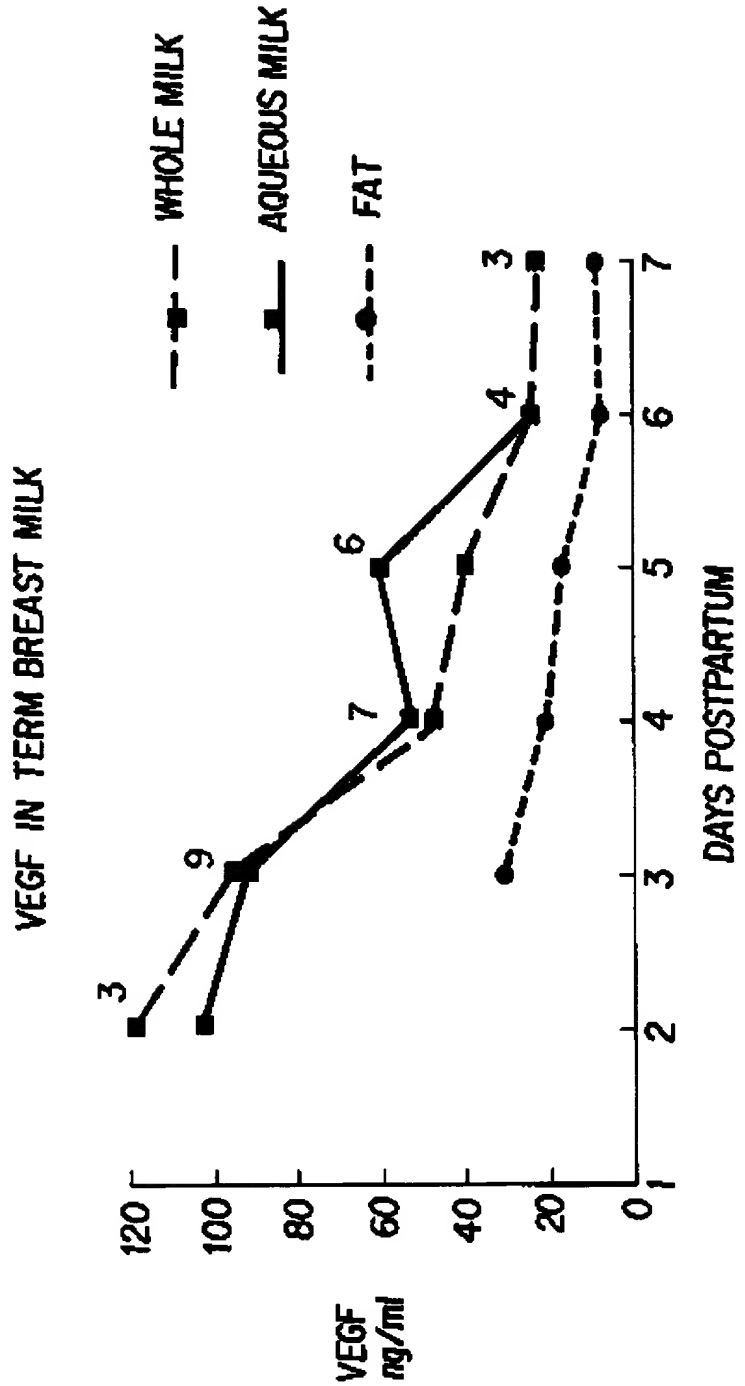
3

FIG. 3


VEGF IN TERM BREAST MILK
WHOLE MILK
AQUEOUS MILK
FAT
VEGF mg/ml
120
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DAYS POSTPARTUM
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FIG. 3

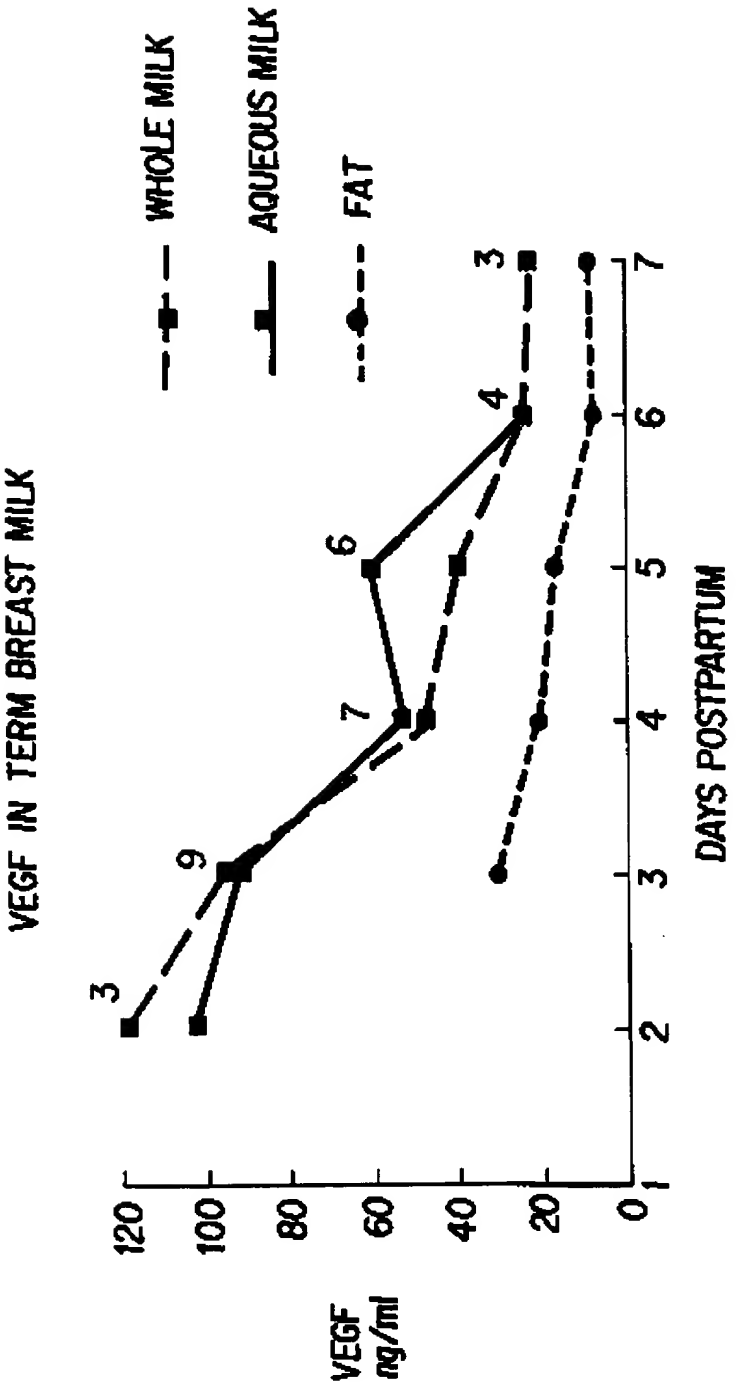
VEGF IN TERM BREAST MILK
WHOLE MILK
AQUEOUS MILK
FAT
VEGF ng/ml
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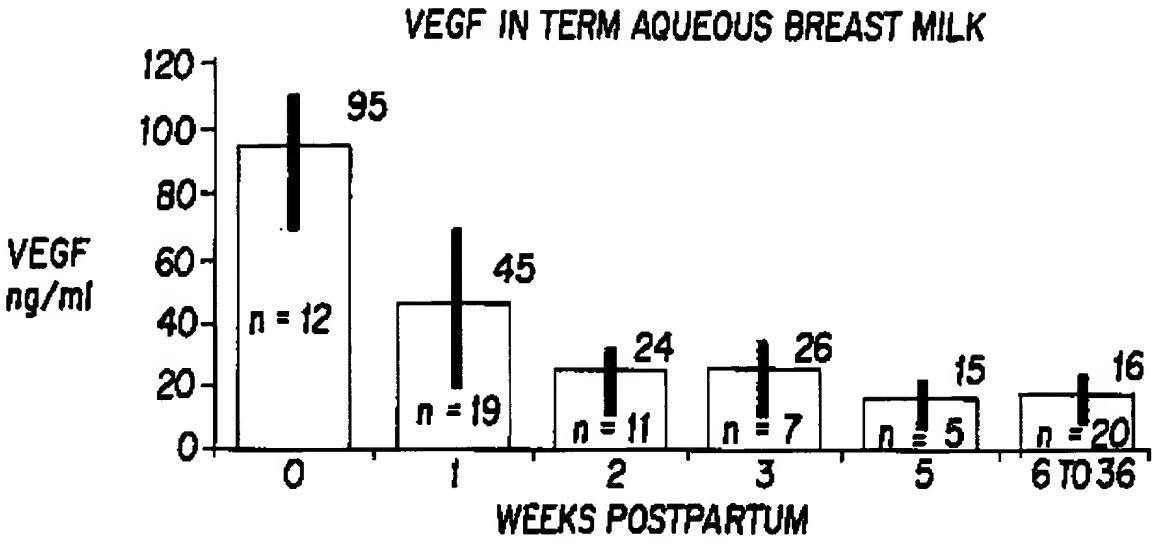
FIG. 3


VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
120
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n = 12
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n = 20
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6 TO 36
WEEKS POSTPARTUM

FIG. 4

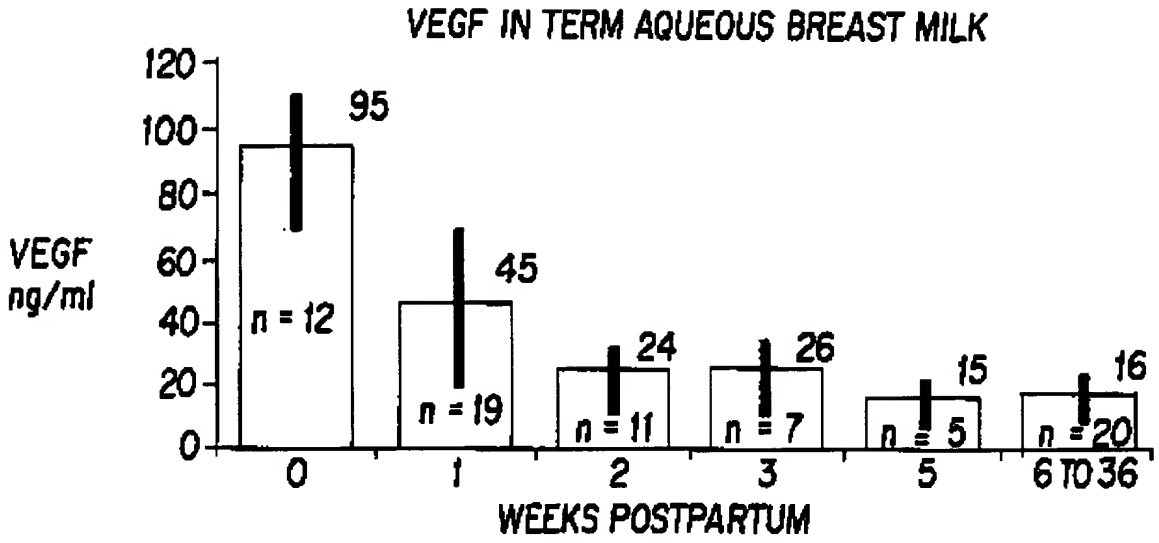
VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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FIG. 4

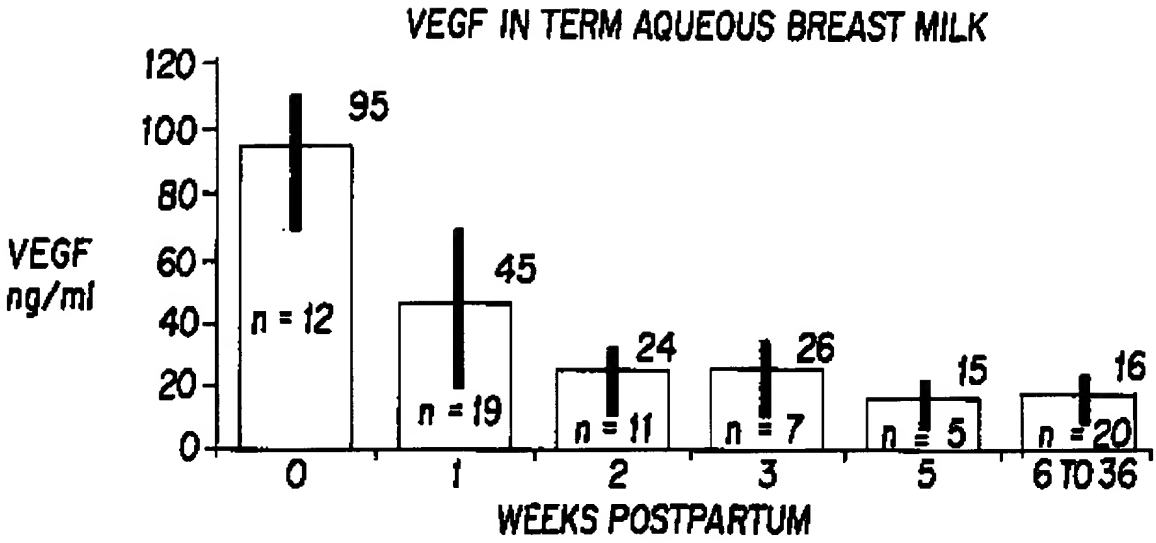
VEGF IN TERM AQUEOUS BREAST MILK
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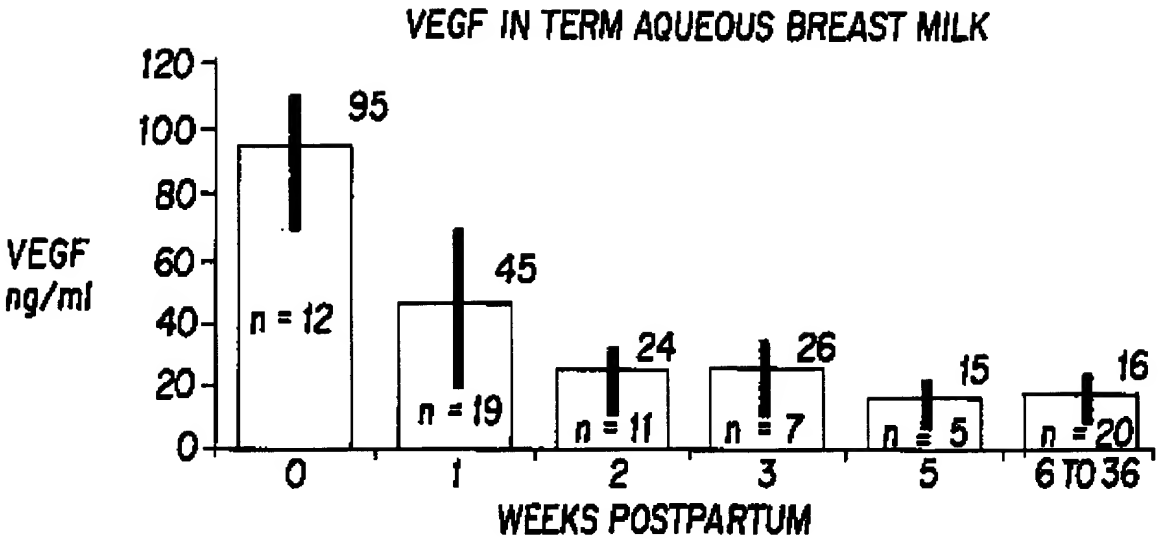
FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
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WEEKS POSTPARTUM

FIG. 4

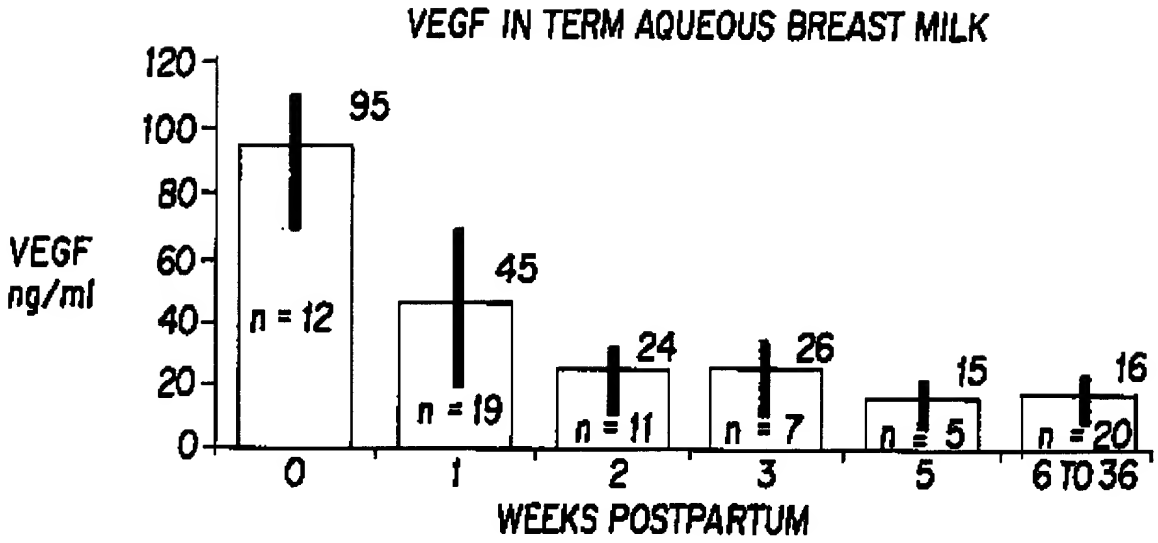
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FIG. 4

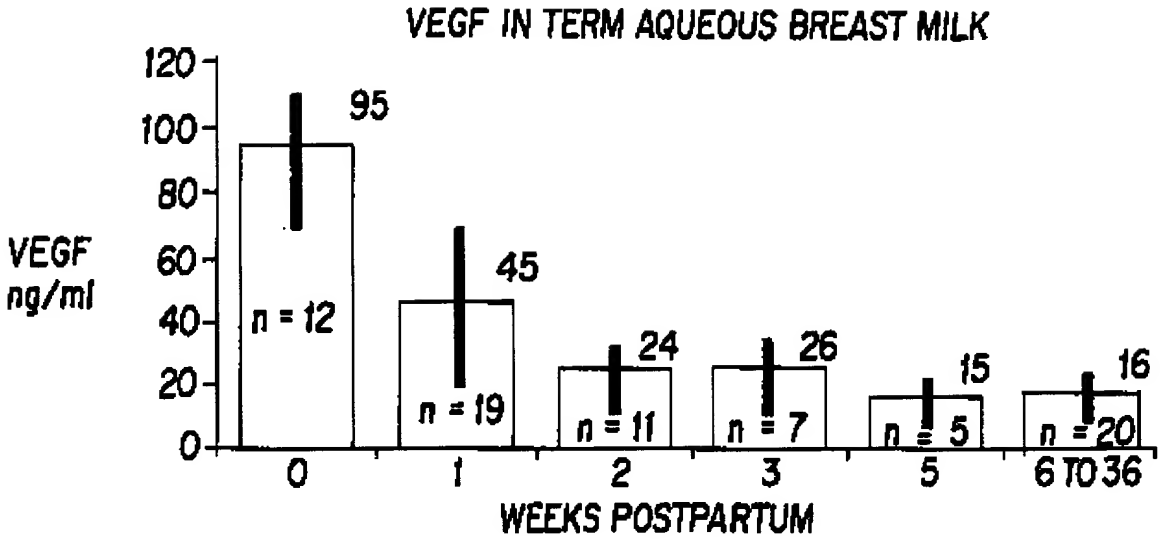
VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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FIG. 4

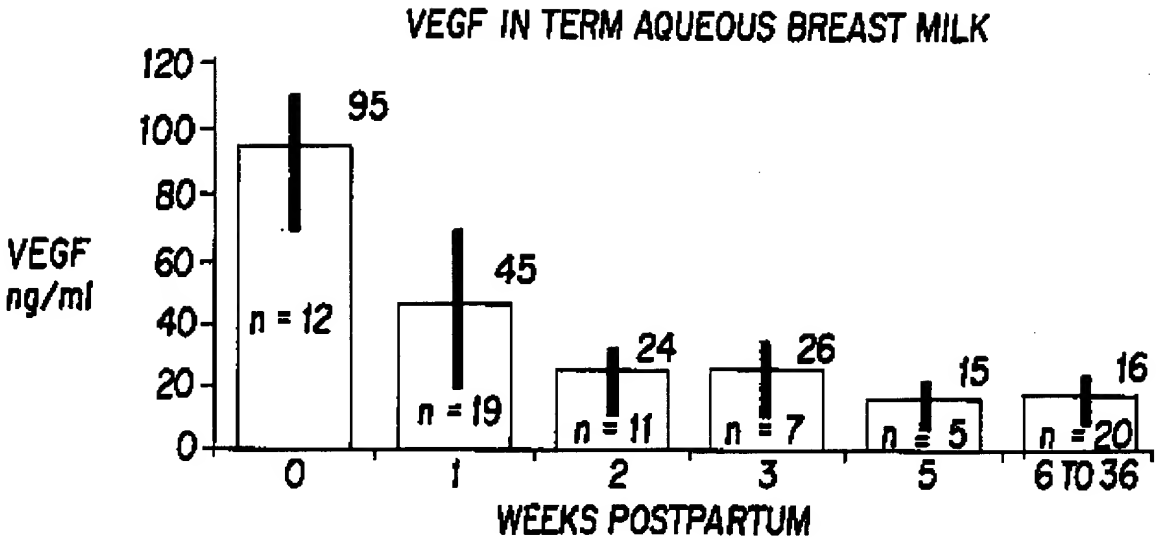
VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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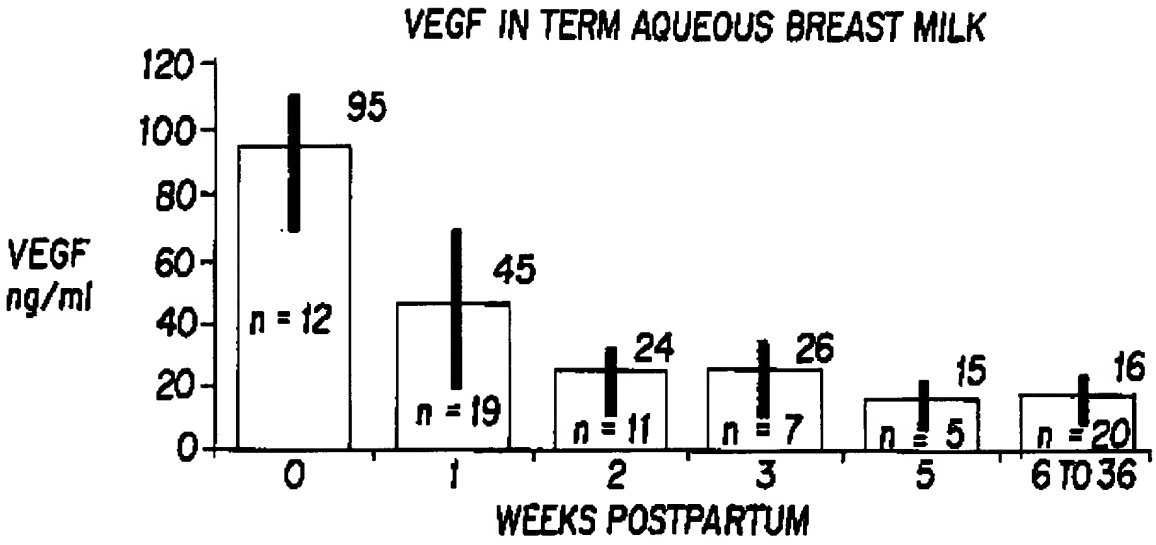
FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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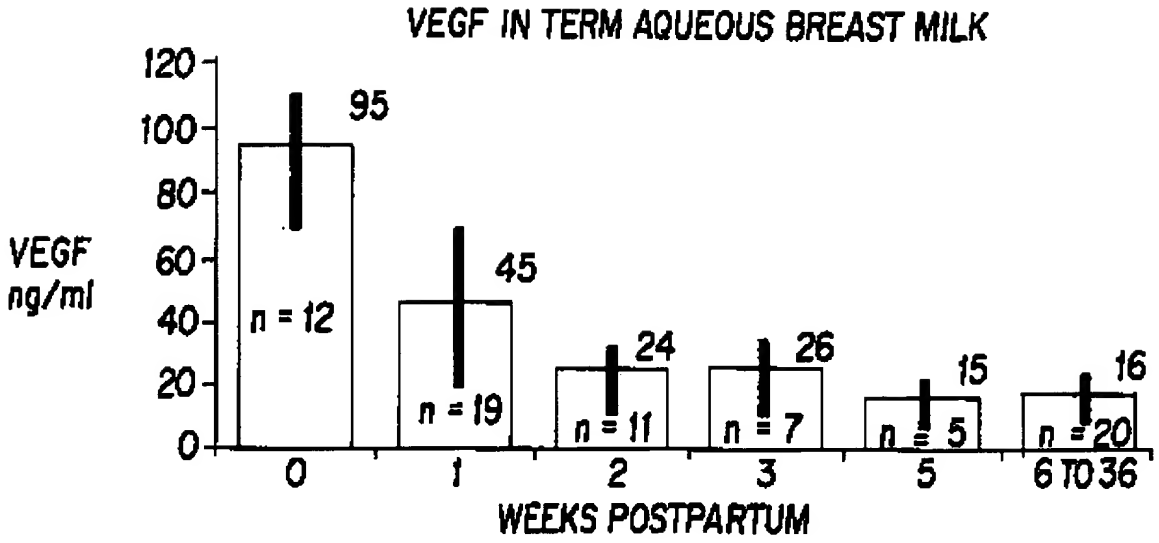
6 TO 36
WEEKS POSTPARTUM

FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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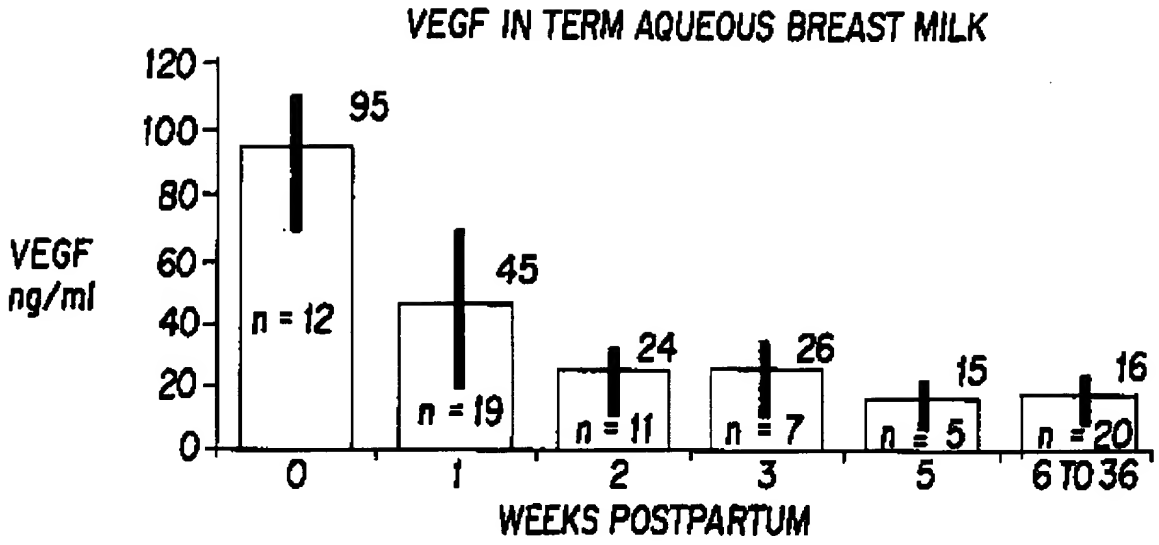
6 TO 36
WEEKS POSTPARTUM

FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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n = 12
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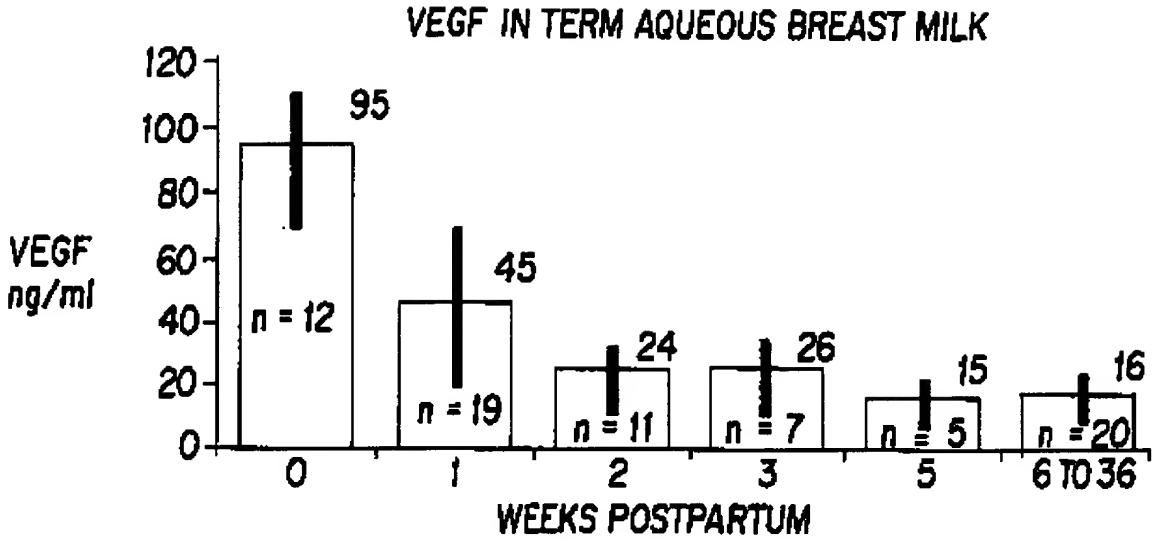
6 TO 36
WEEKS POSTPARTUM

FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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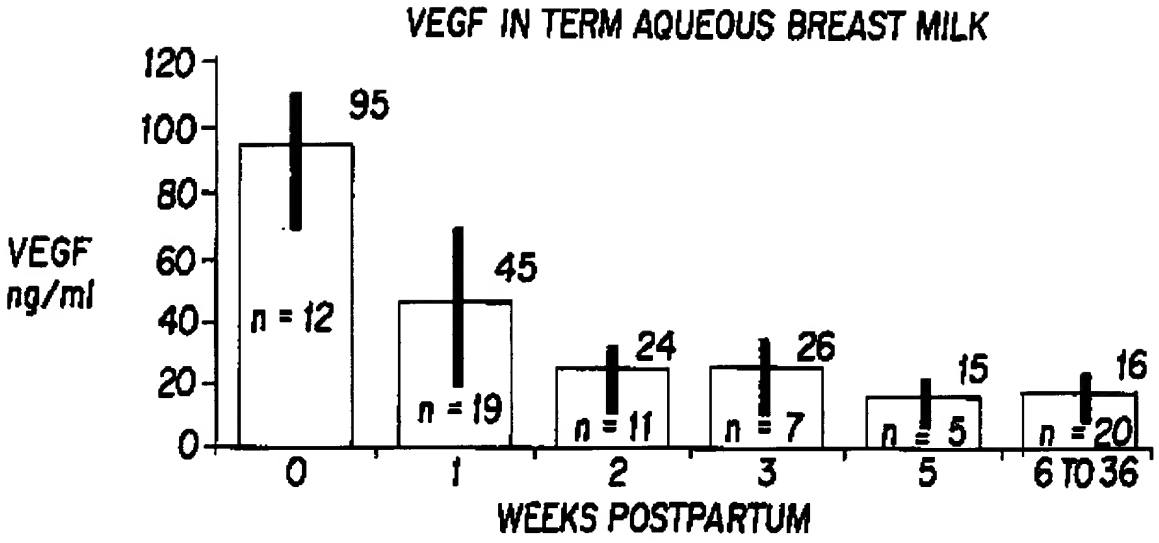
6 TO 36
WEEKS POSTPARTUM

FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
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6 TO 36
WEEKS POSTPARTUM

FIG. 4

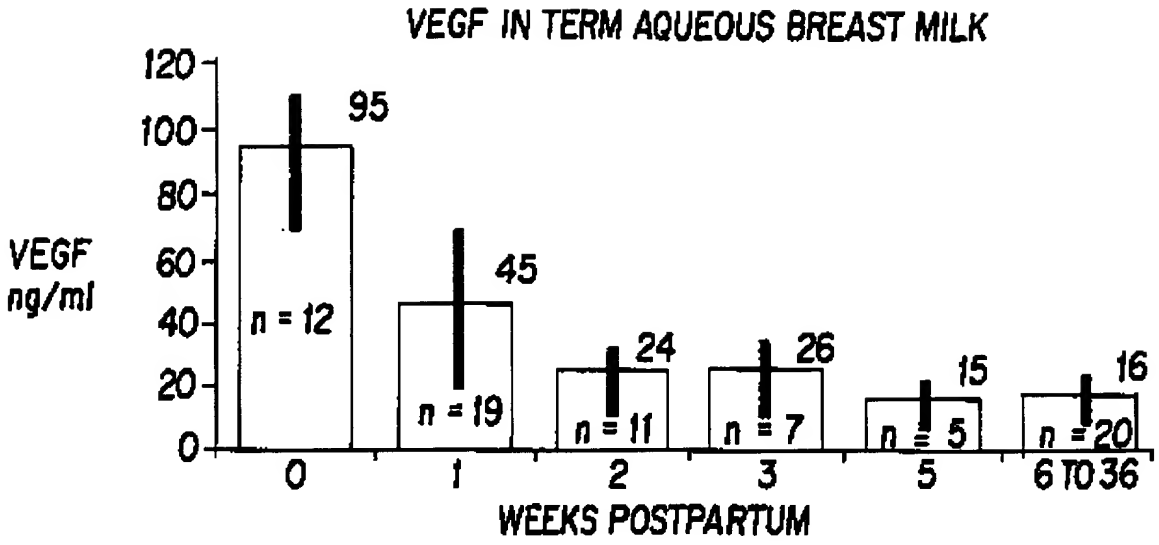
VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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FIG. 4

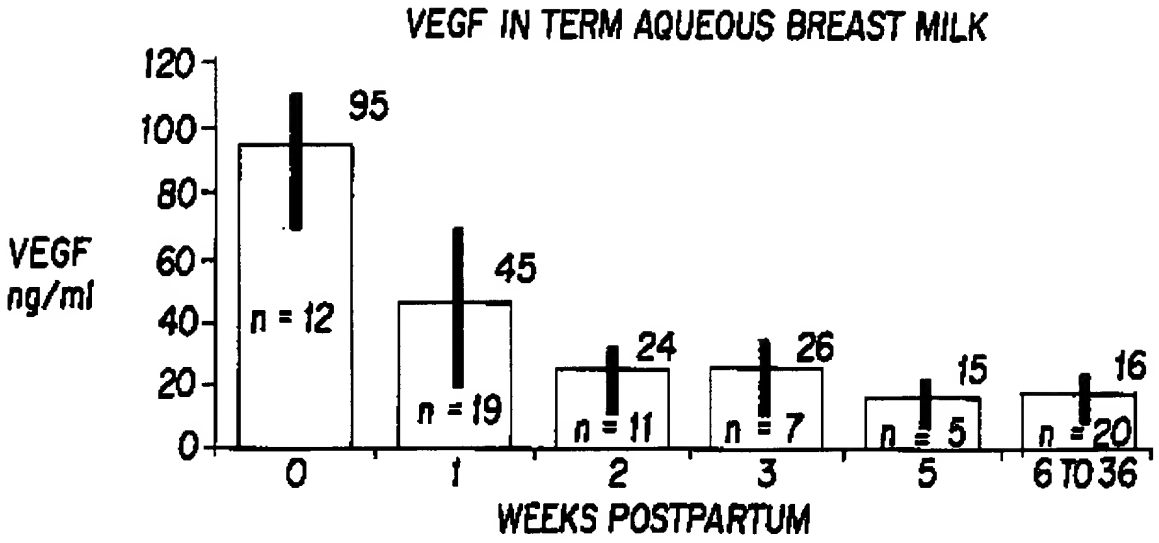
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FIG. 4

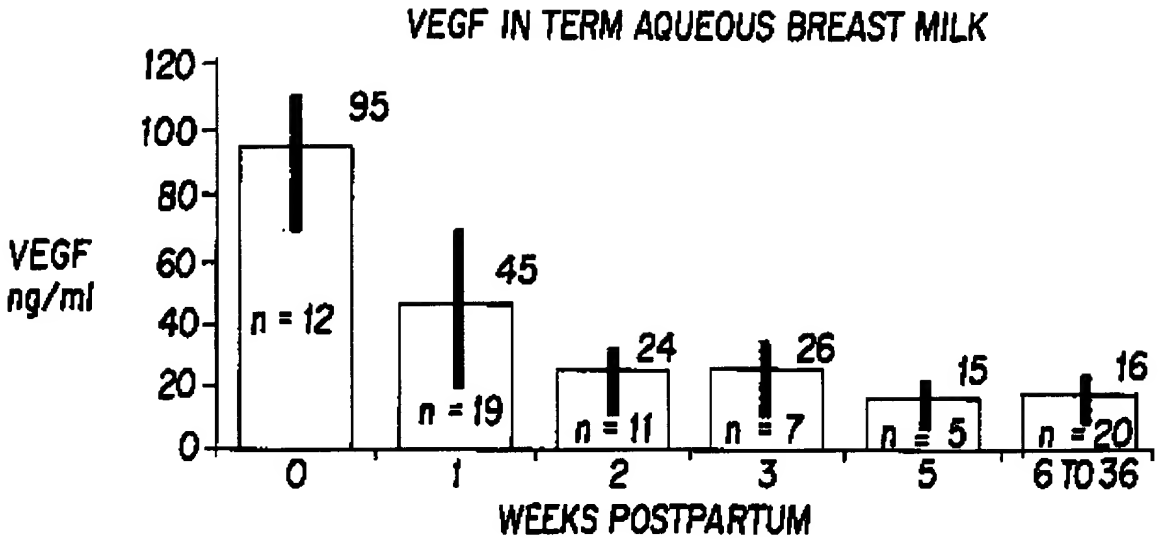
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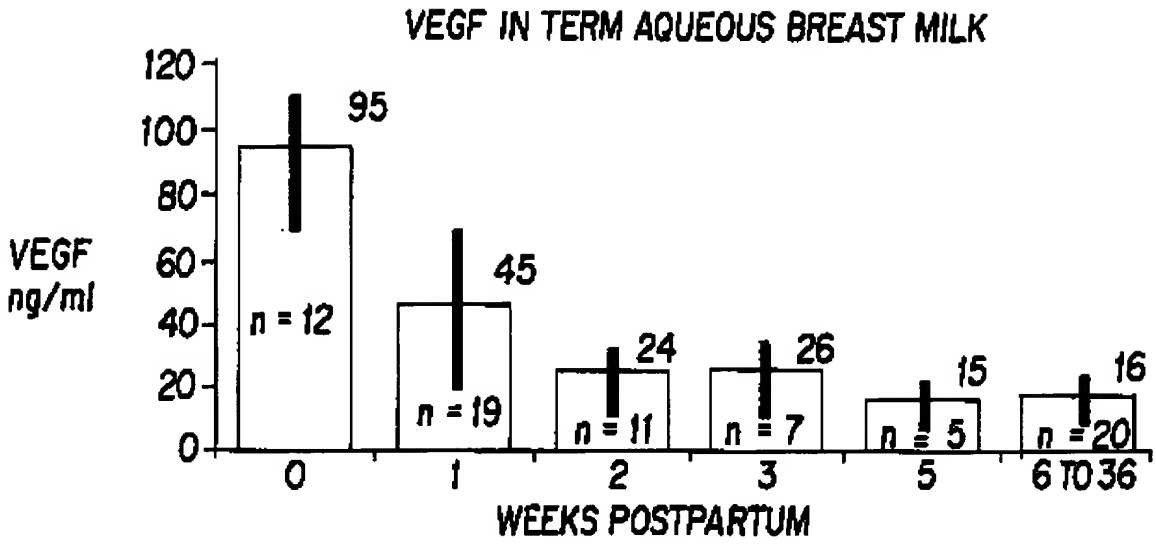
FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
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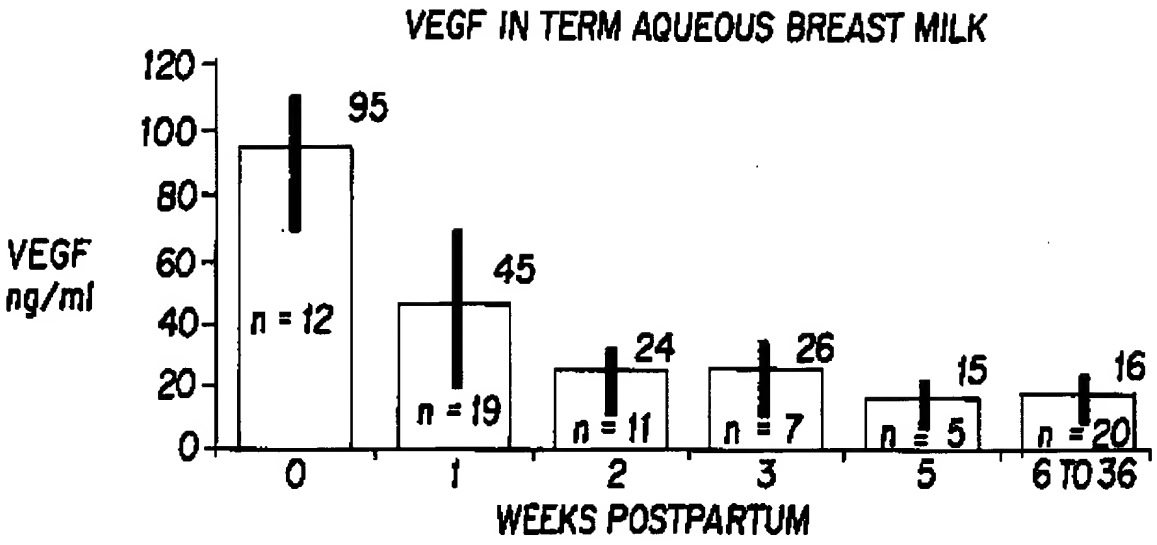
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VEGF ng/ml
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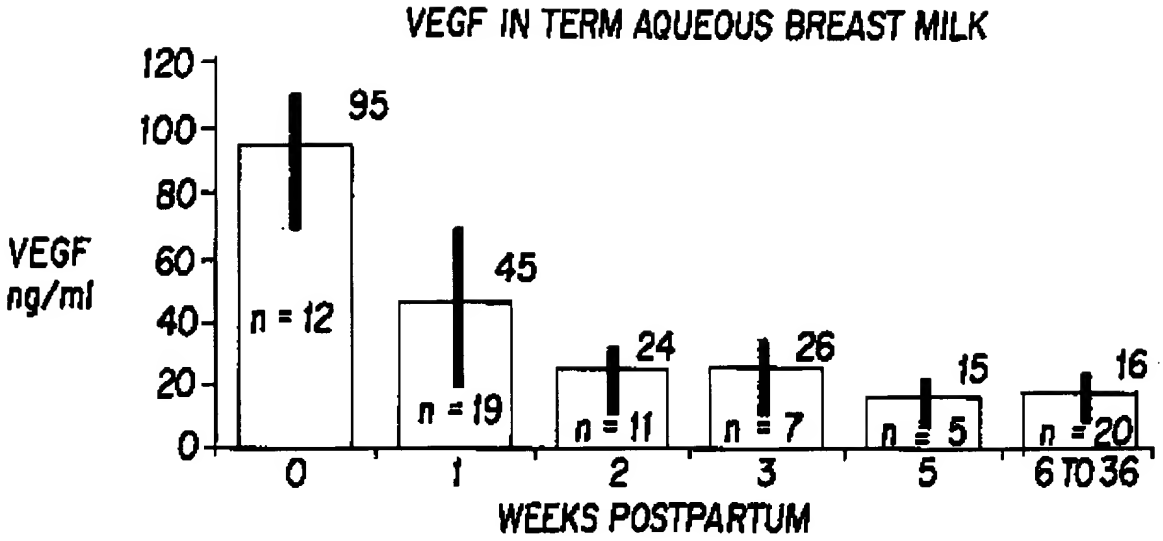
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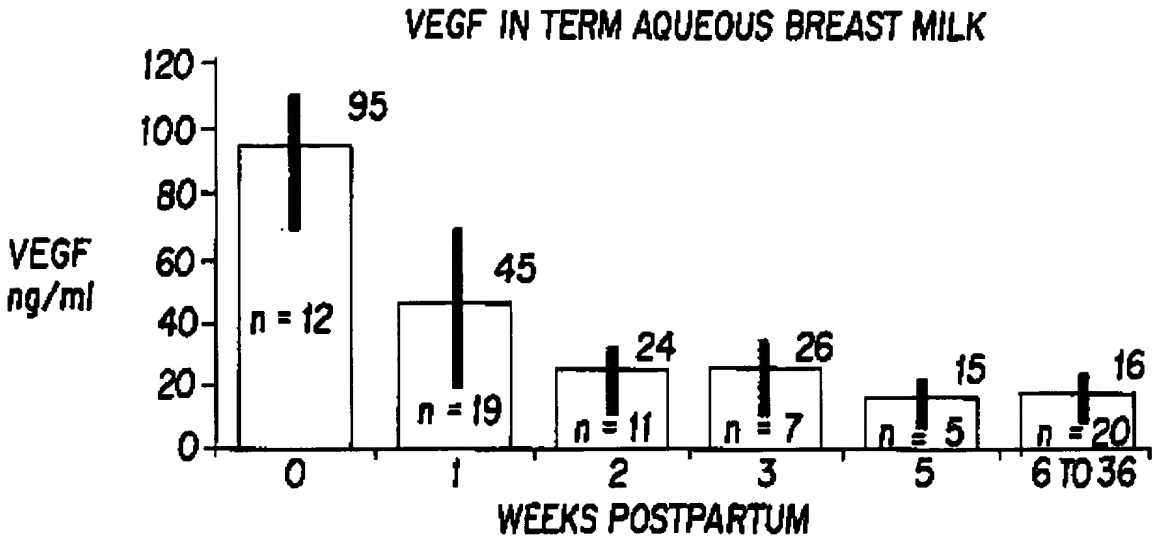
FIG. 4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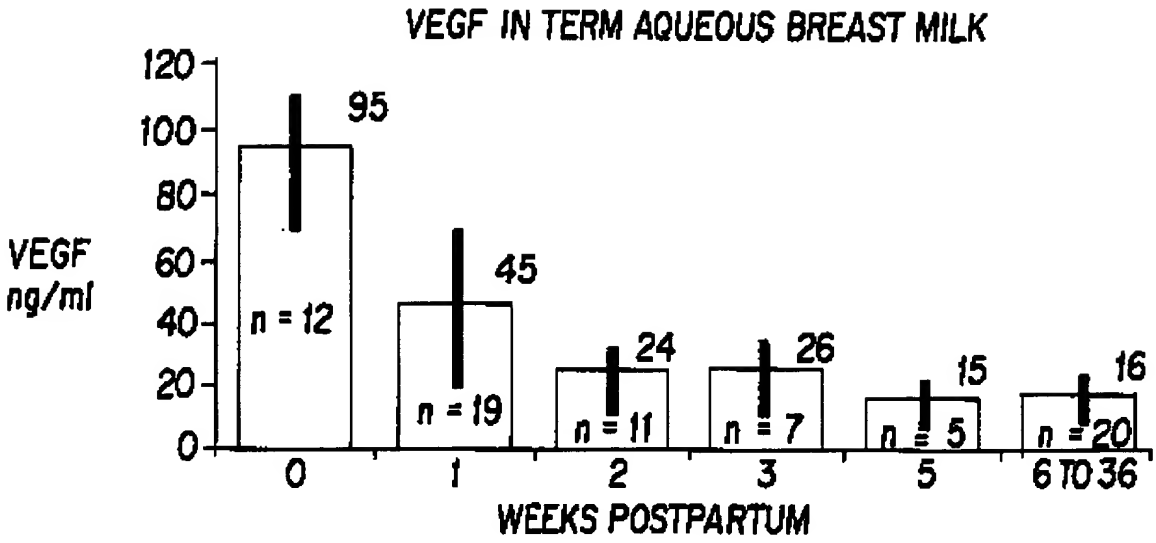
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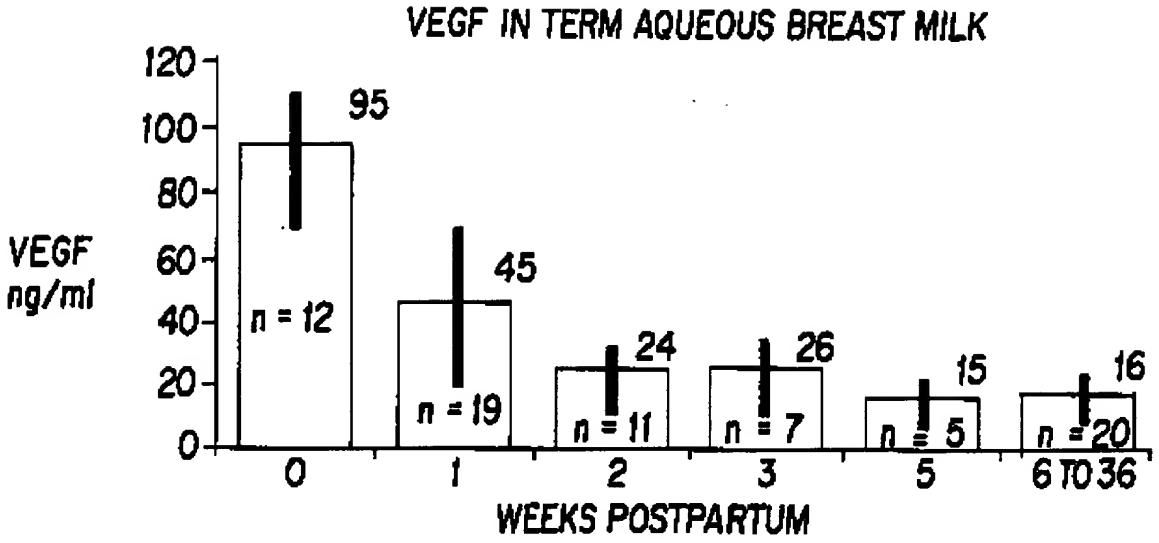
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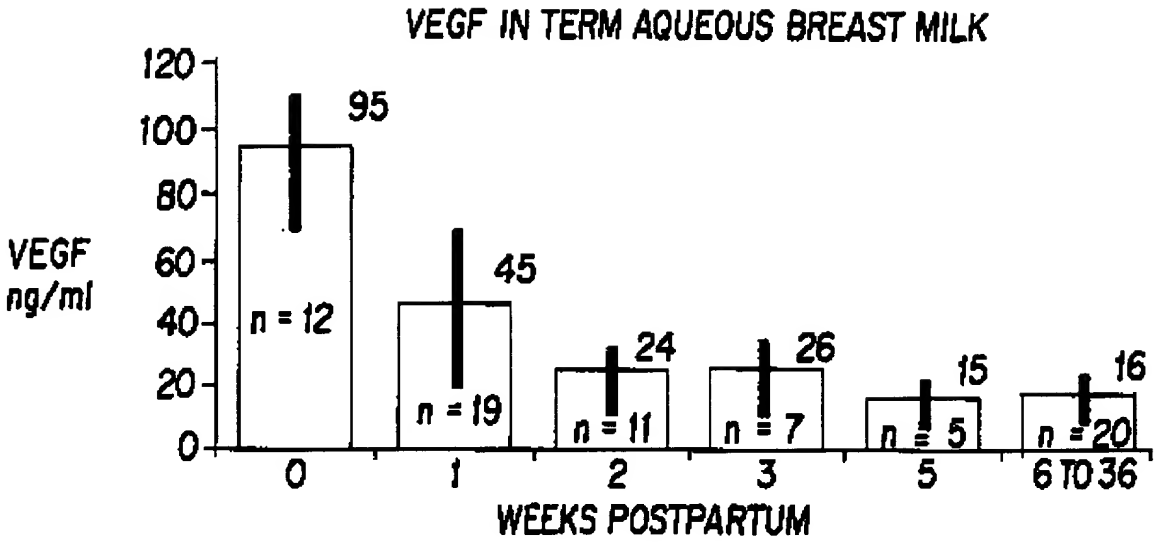
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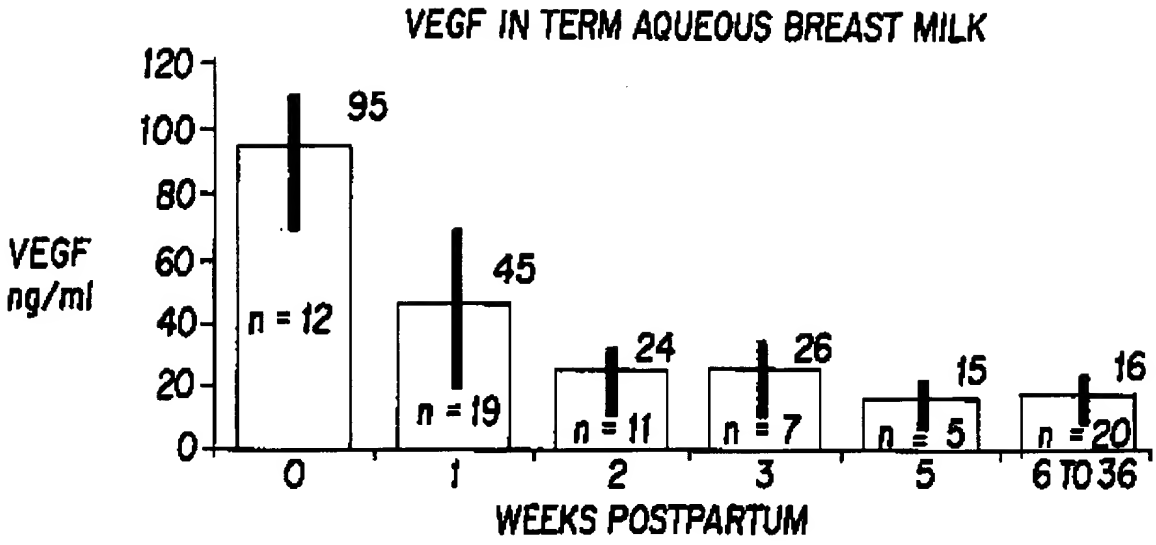
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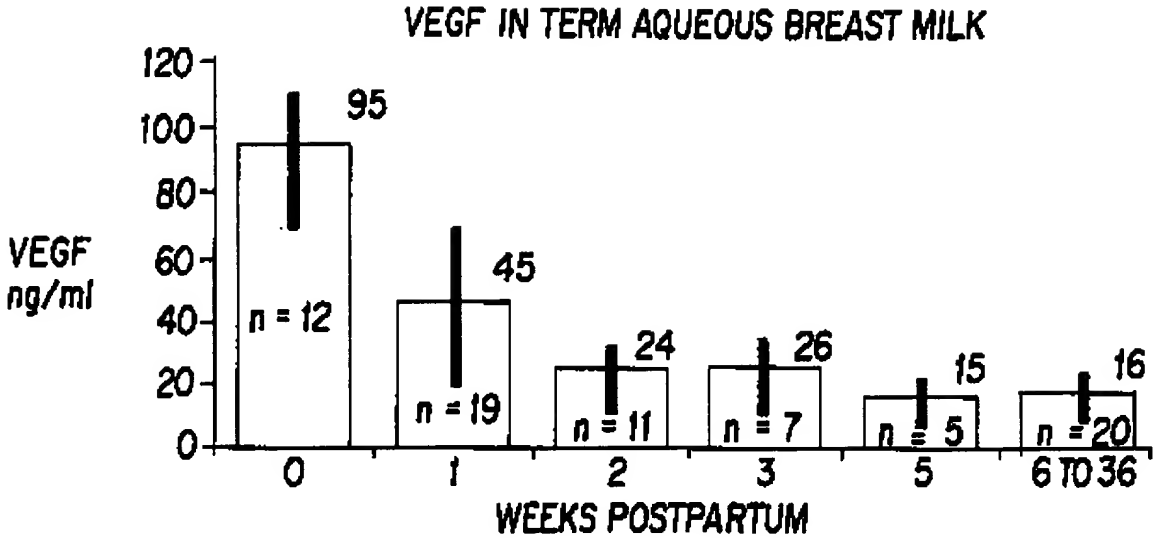
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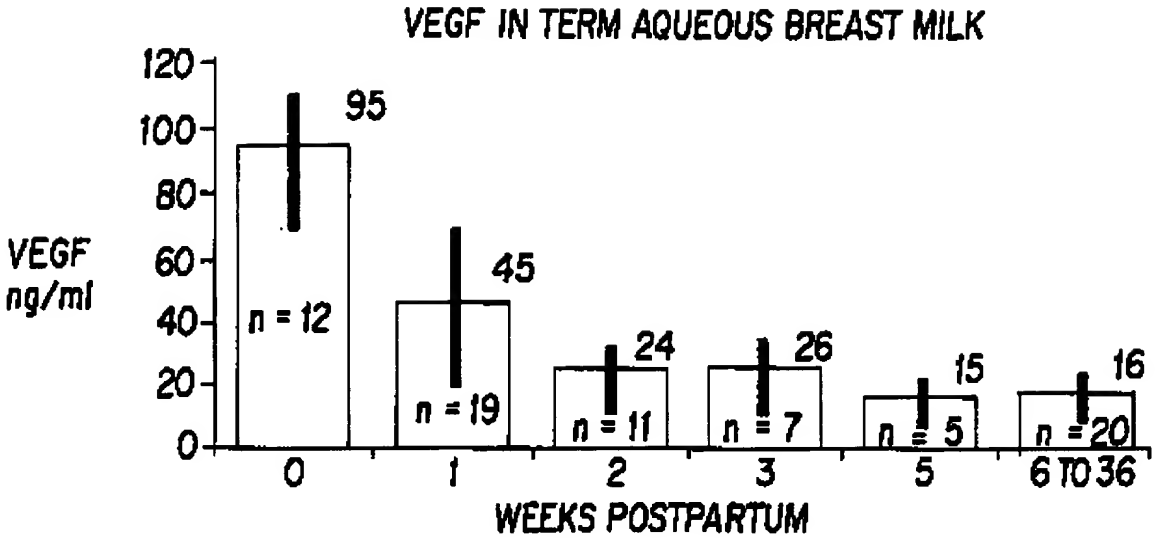
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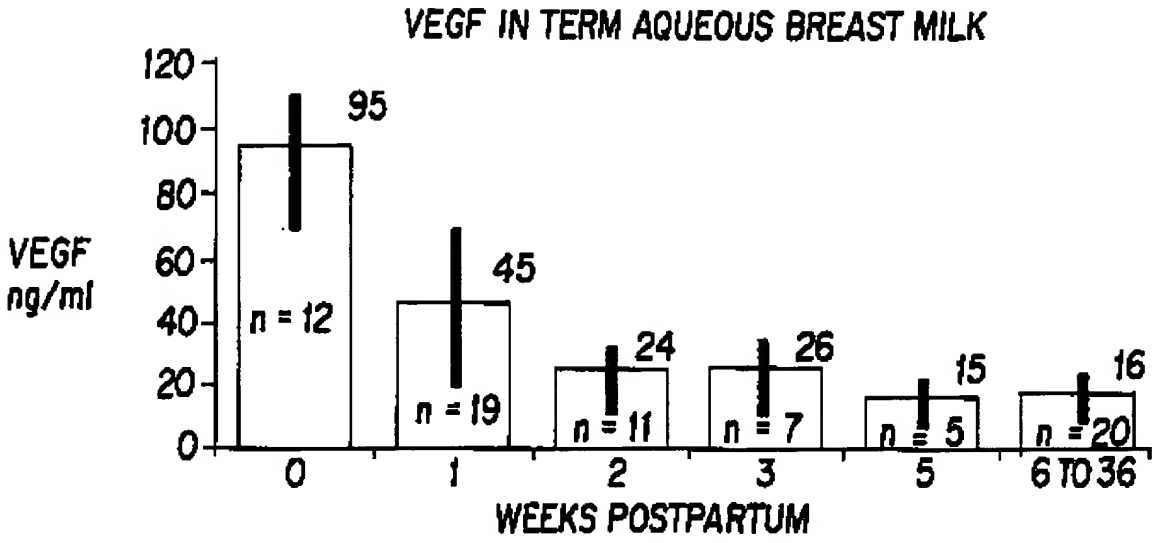
VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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6 TO 36
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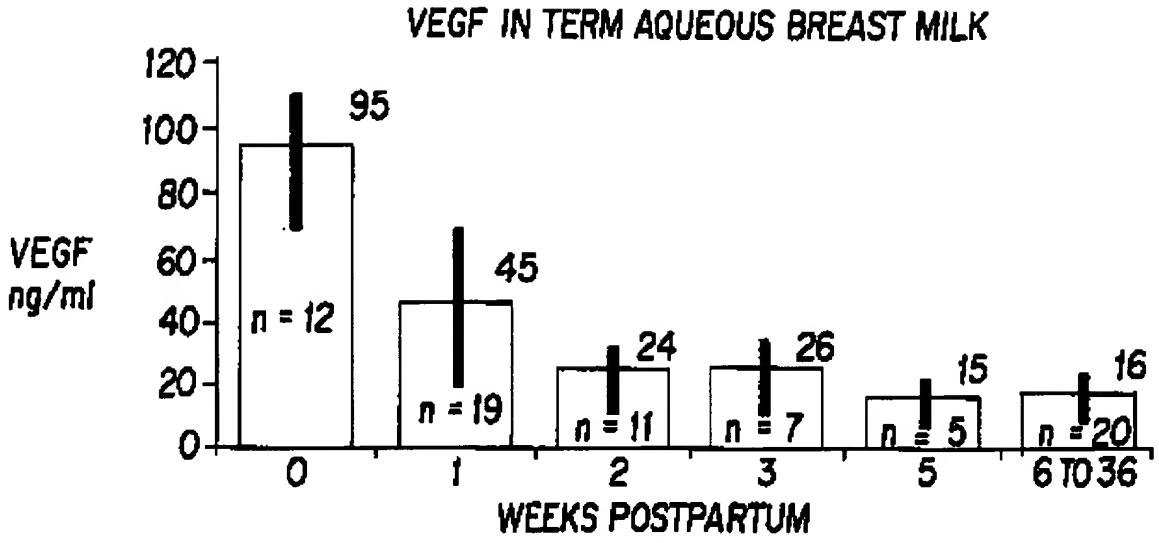
VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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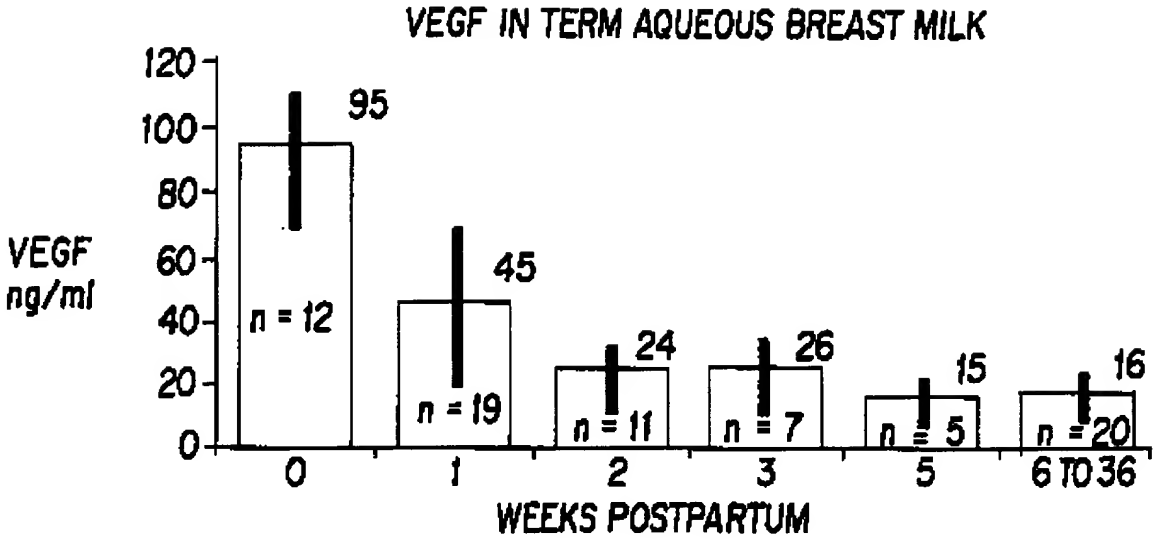
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VEGF ng/ml
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6 TO 36
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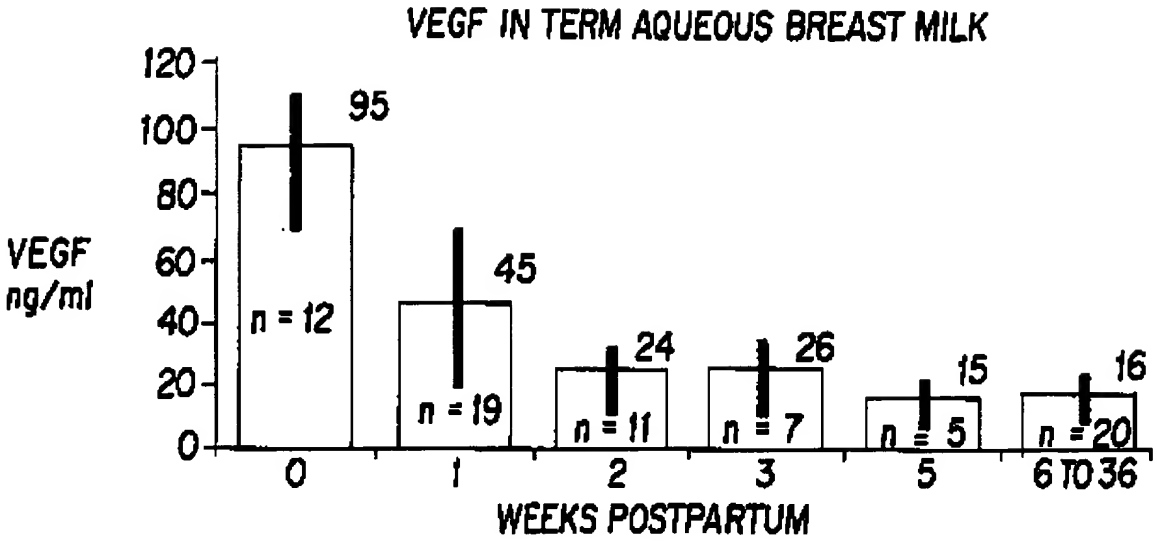
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VEGF ng/ml
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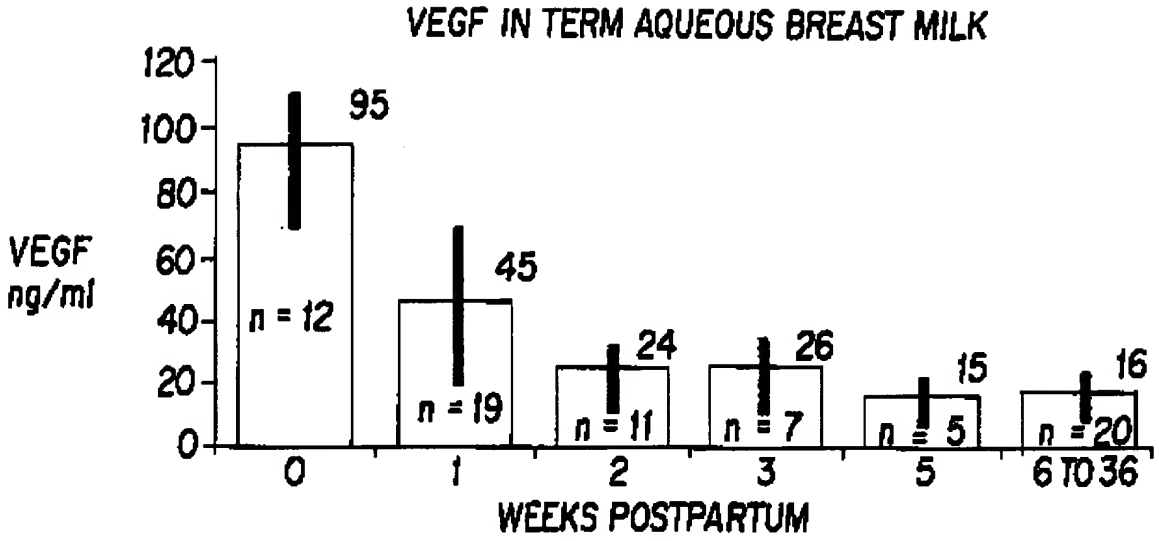
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VEGF ng/ml
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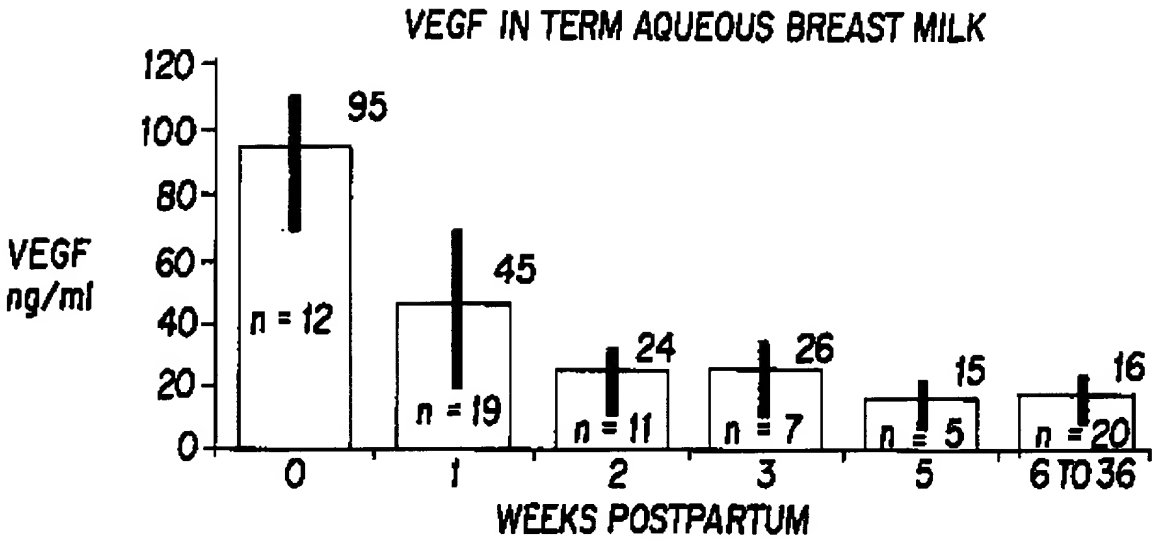
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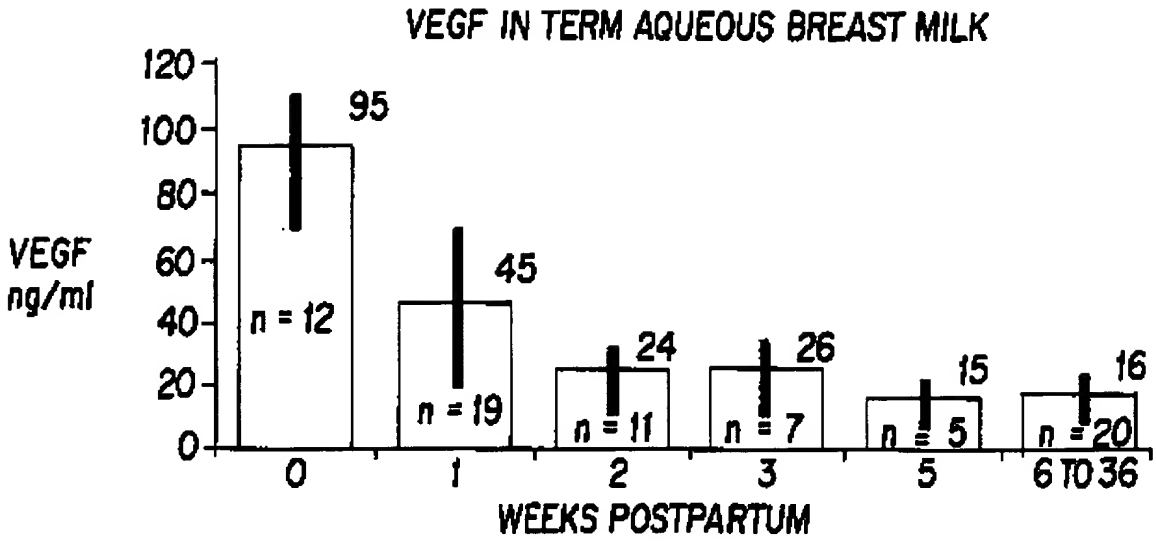
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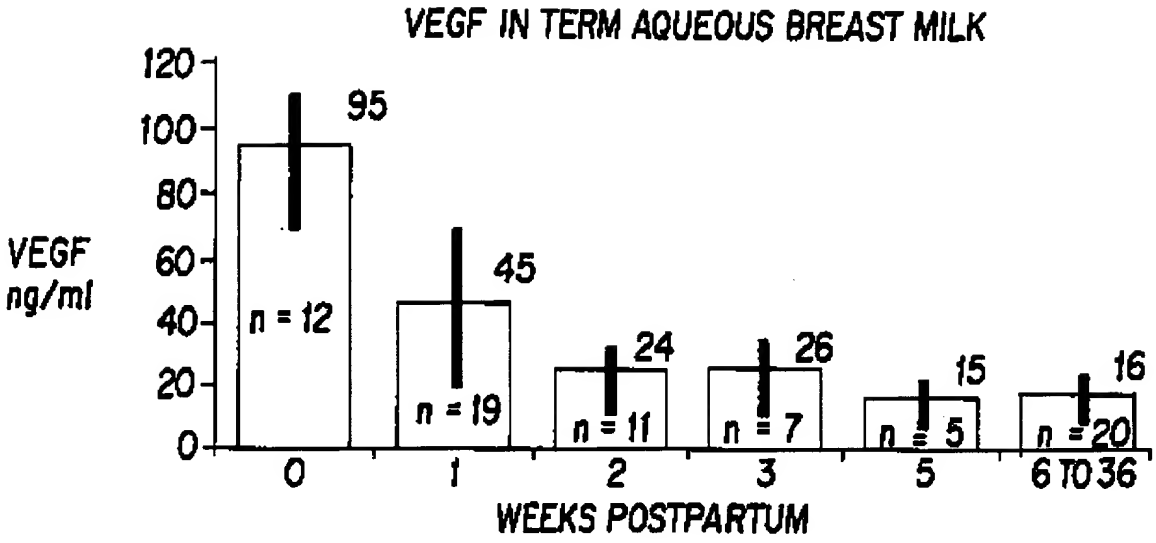
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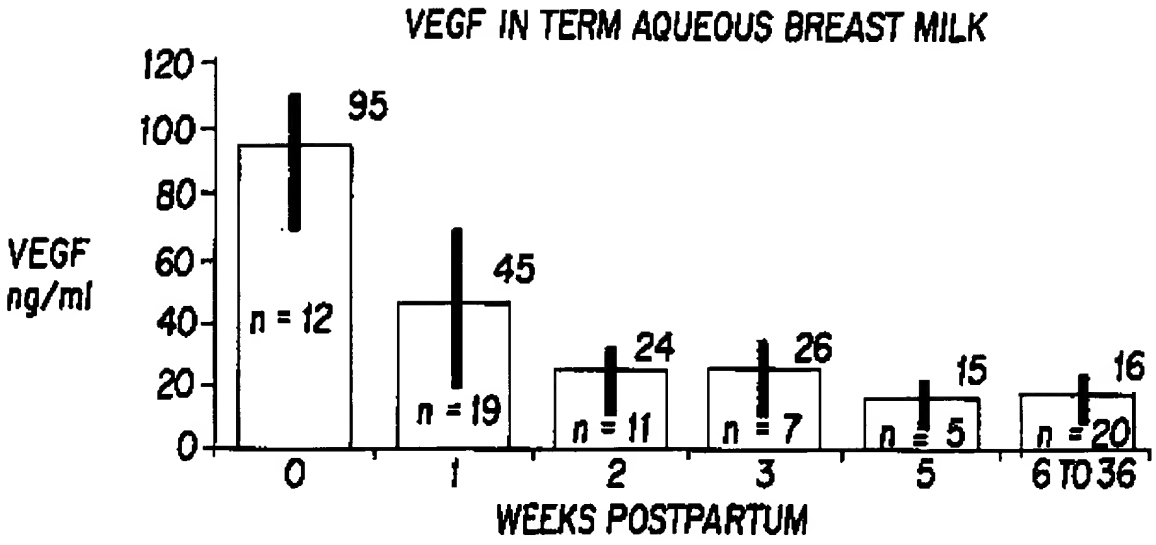
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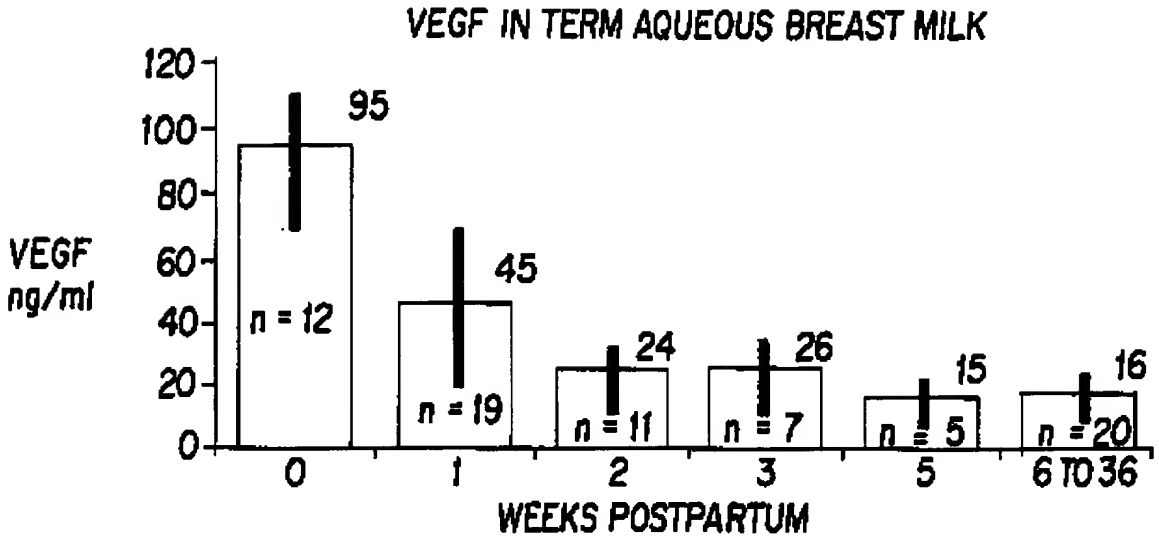
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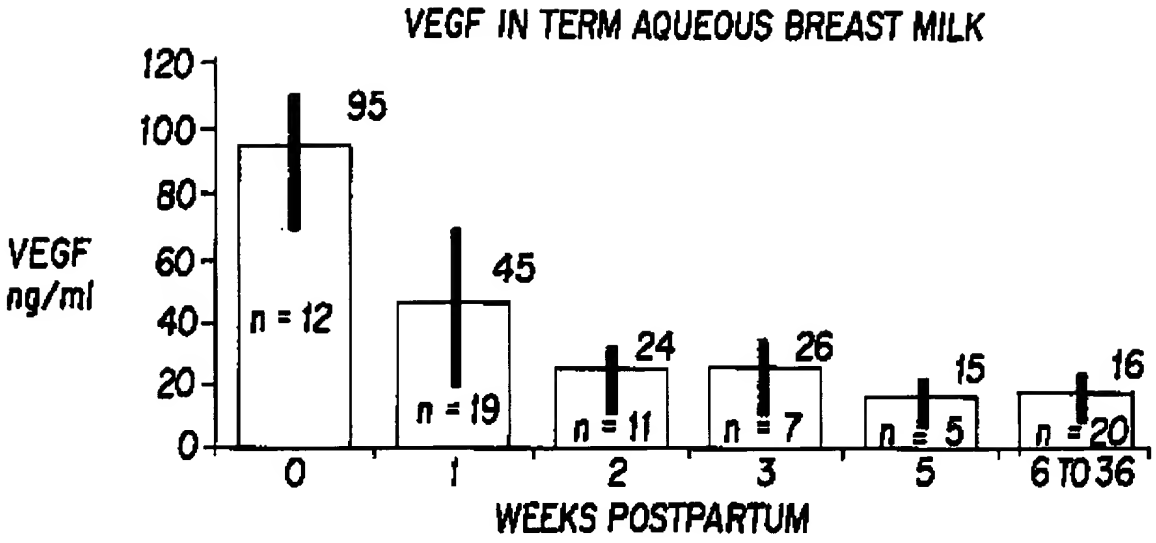
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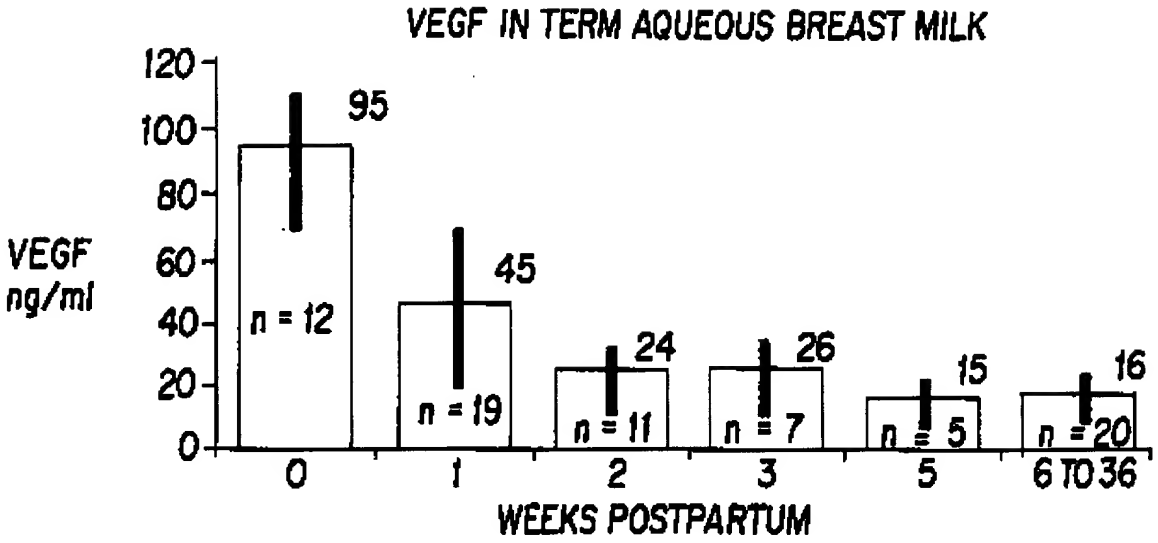
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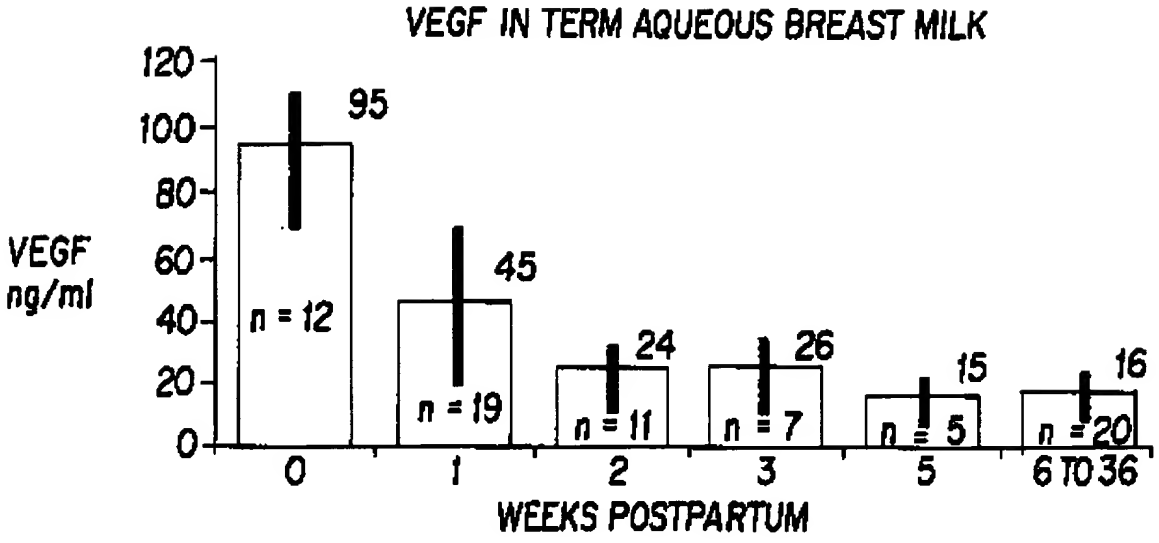
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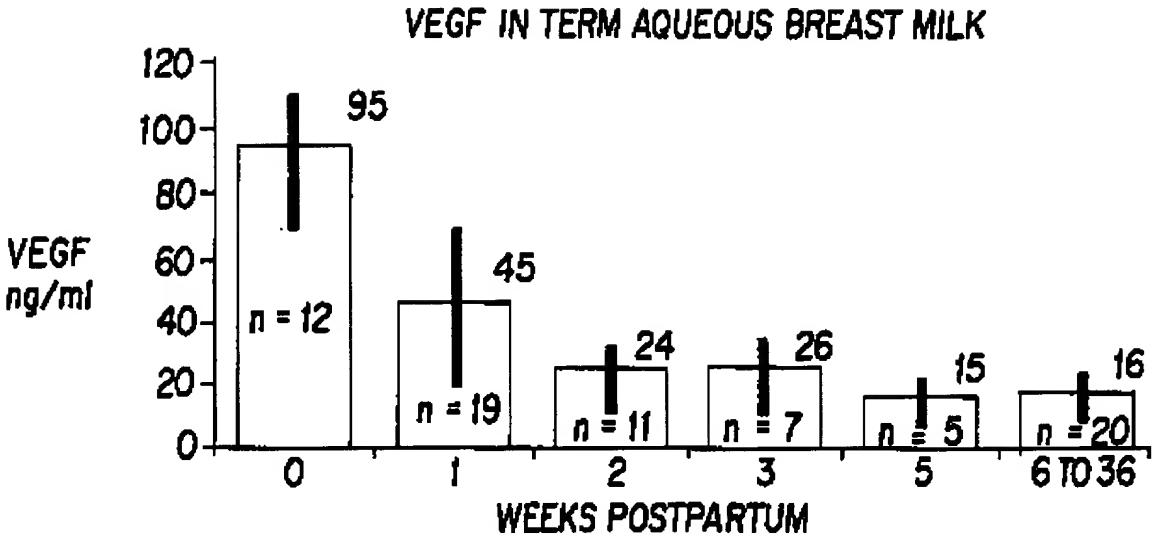
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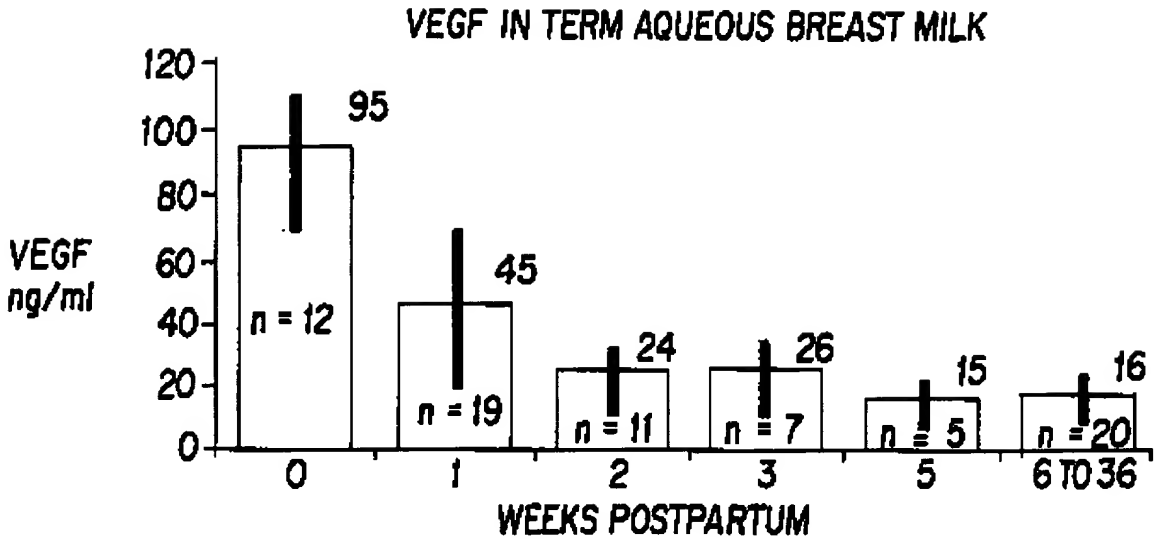
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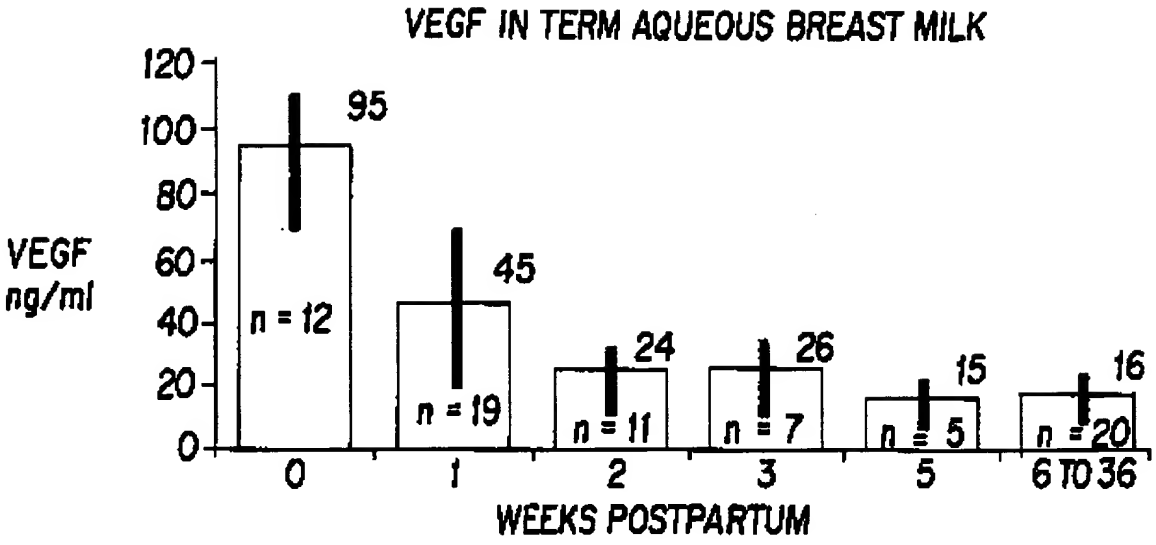
WEEKS POSTPARTUM

FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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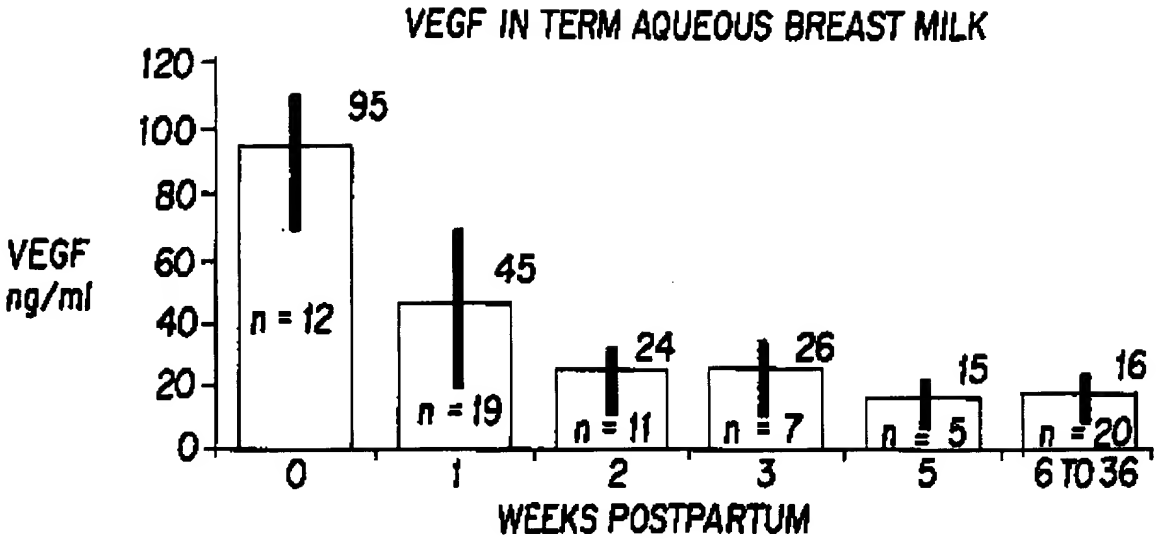
6 TO 36
WEEKS POSTPARTUM

FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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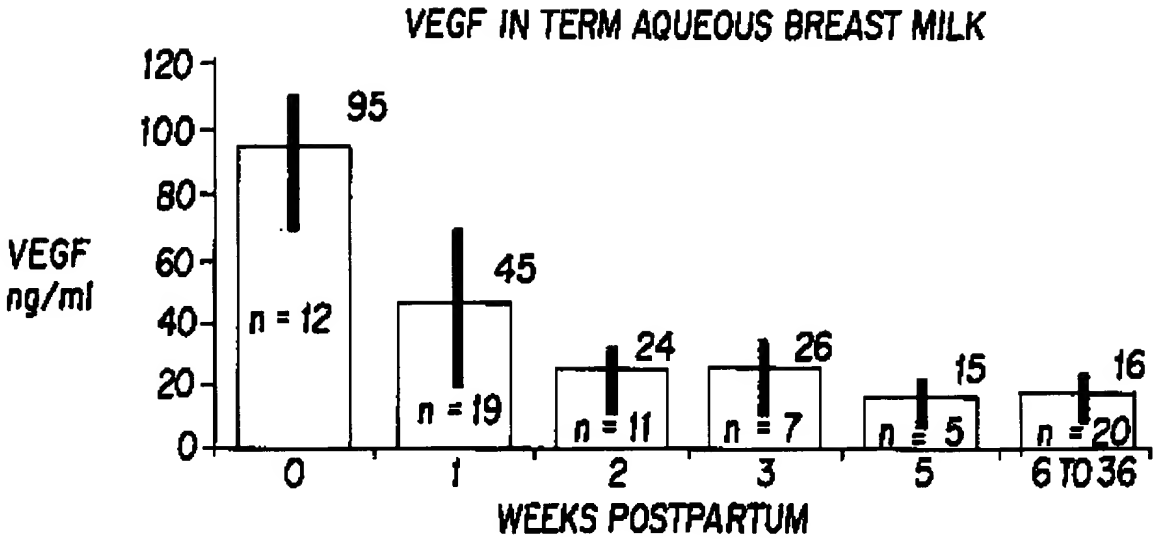
6 TO 36
WEEKS POSTPARTUM

FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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6 TO 36
WEEKS POSTPARTUM

FIG. 4

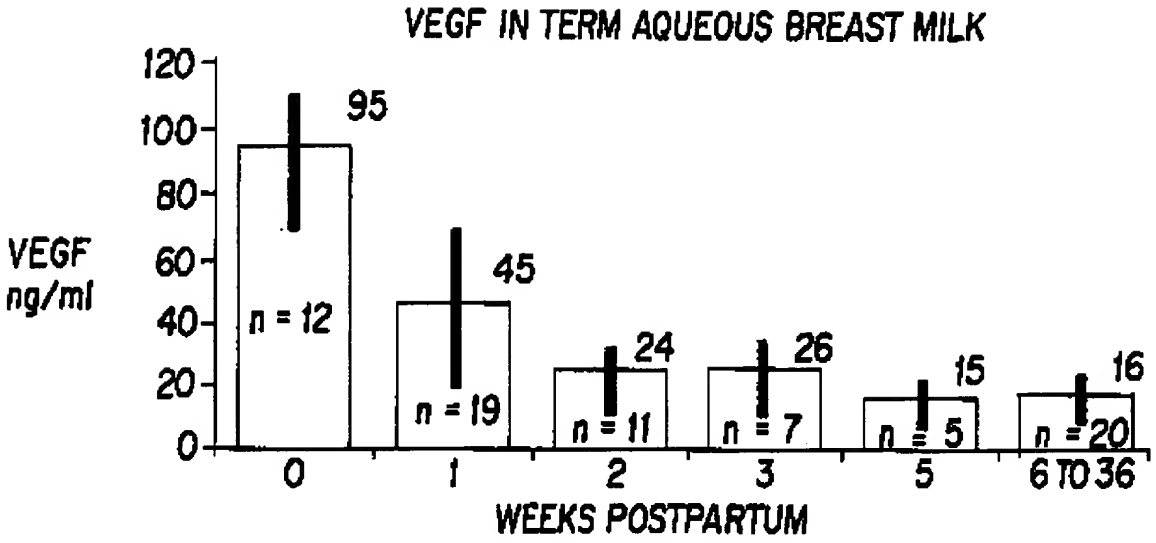
VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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FIG. 4

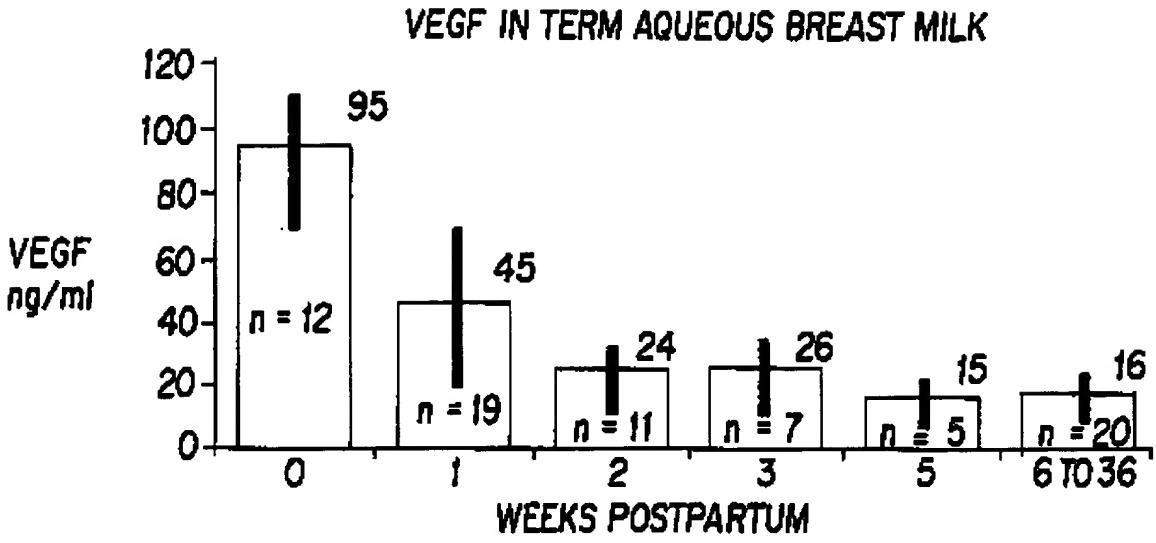
VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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FIG. 4

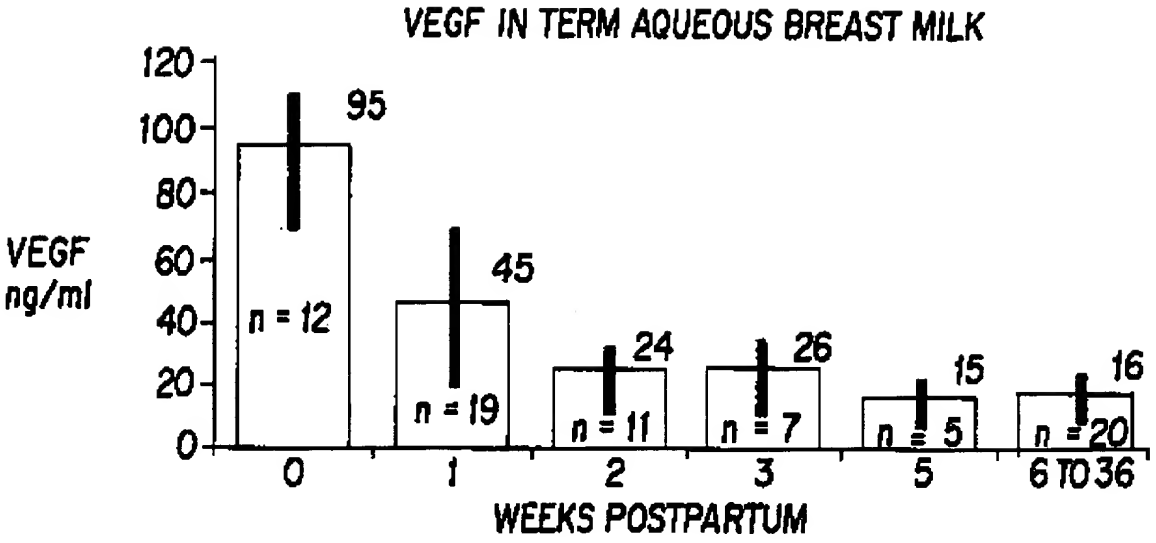
VEGF IN TERM AQUEOUS BREAST MILK
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FIG. 4

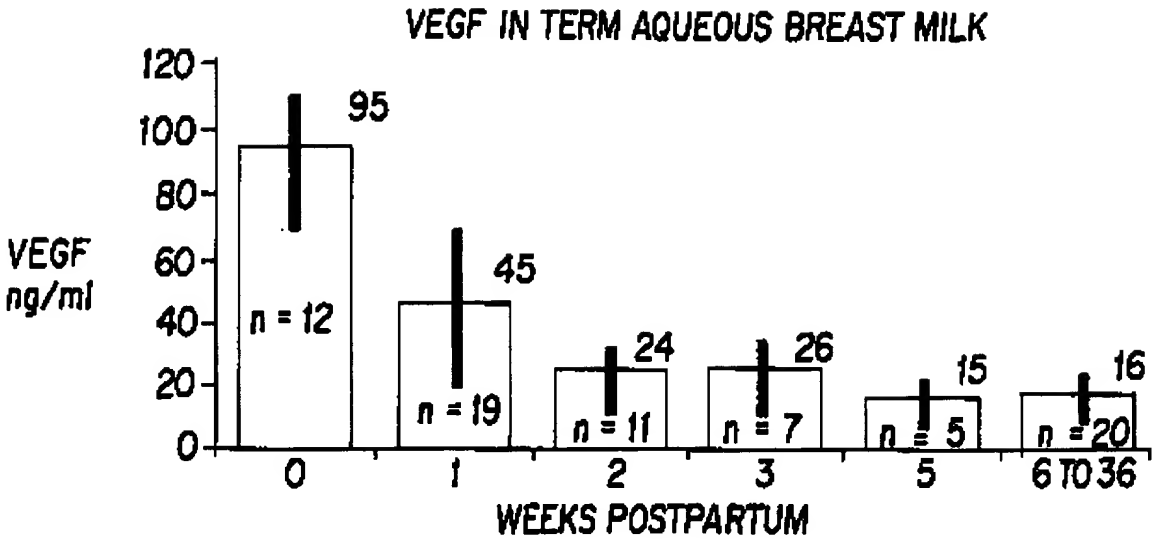
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FIG. 4

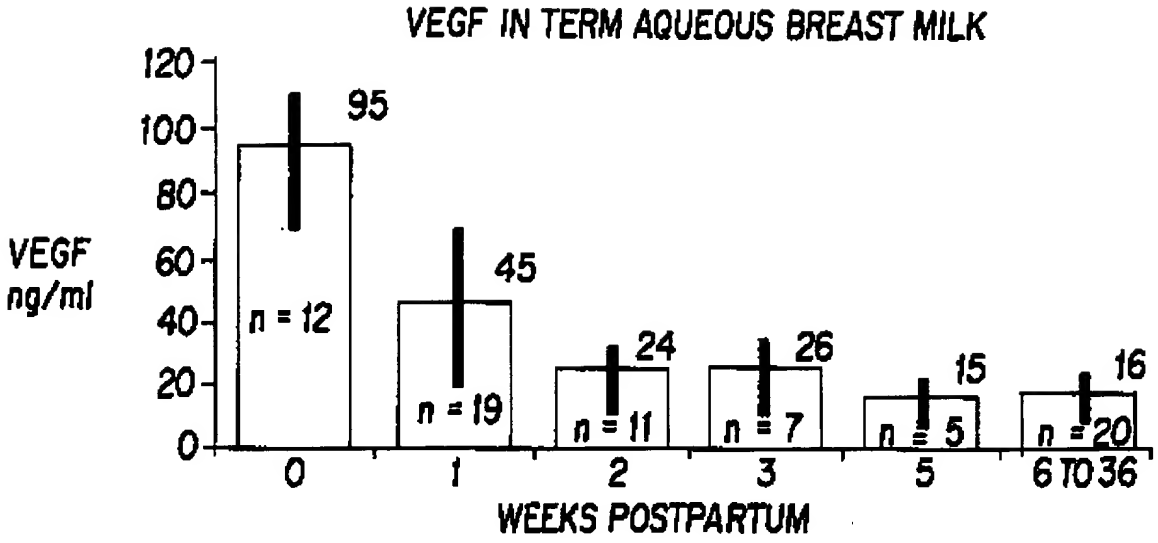
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FIG. 4

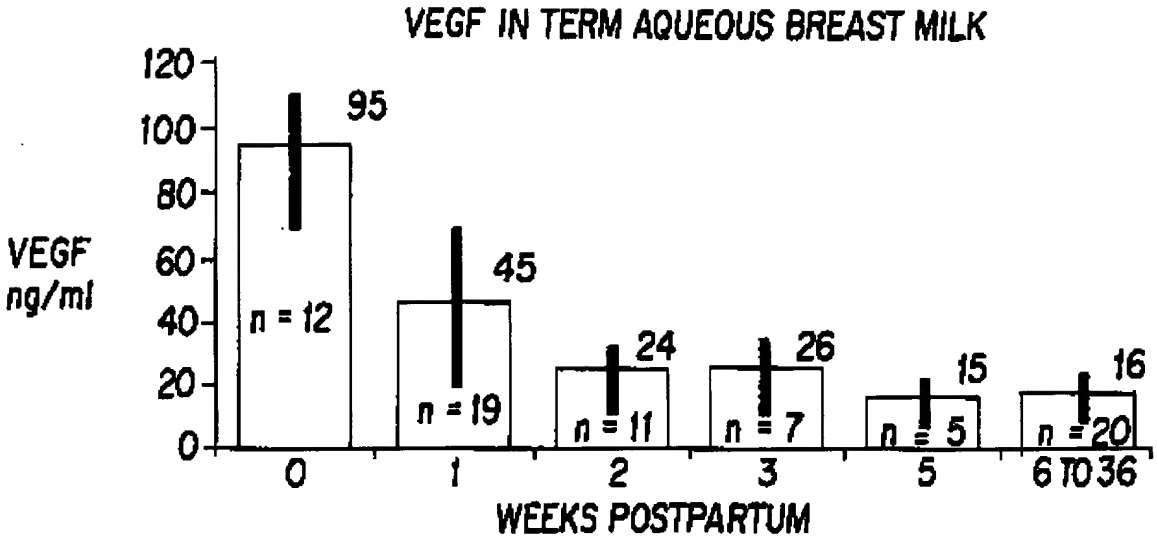
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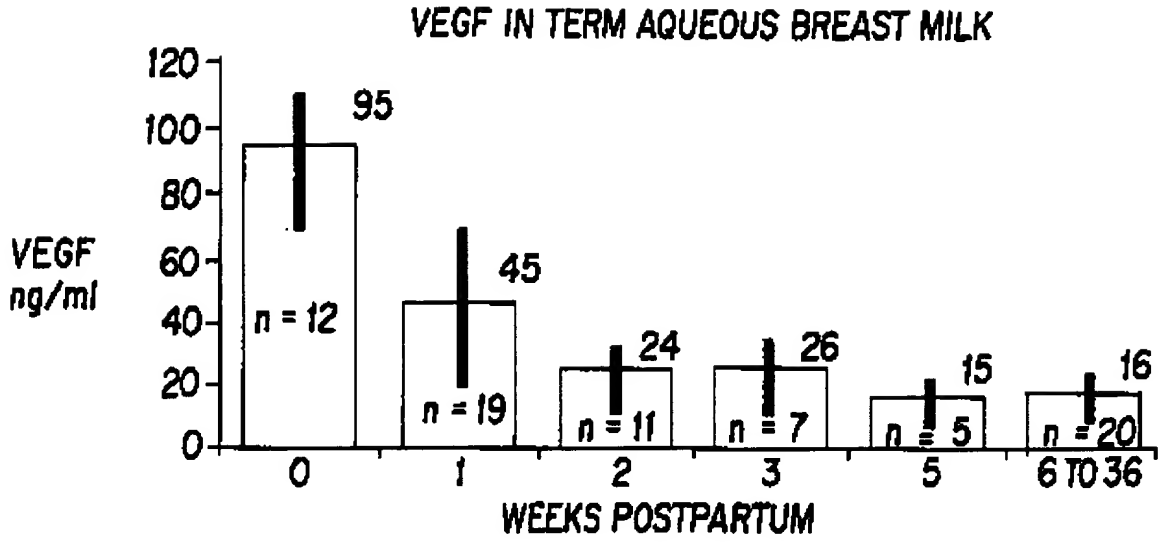
FIG. 4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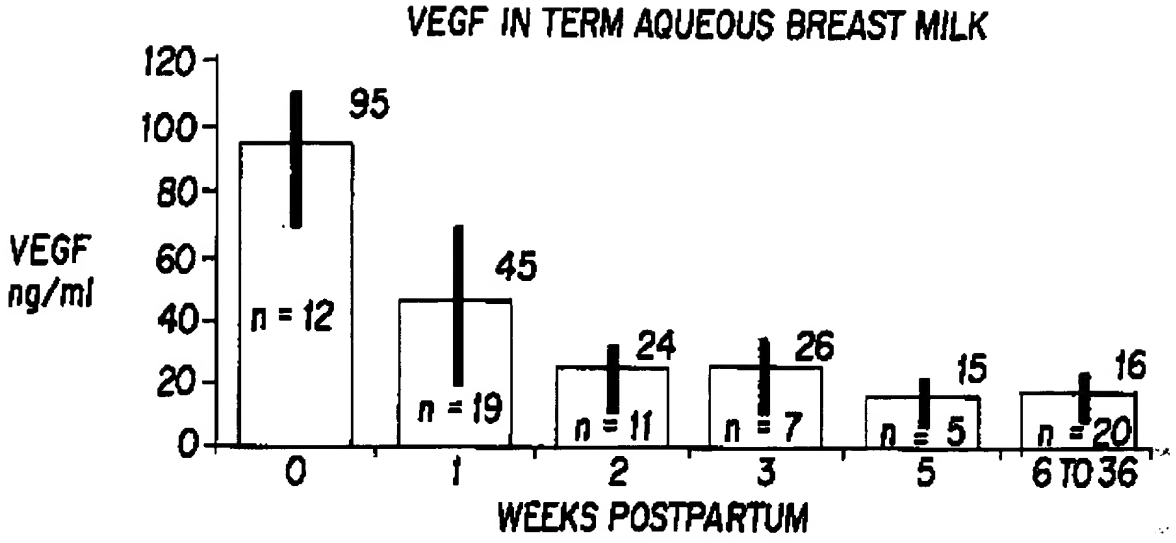
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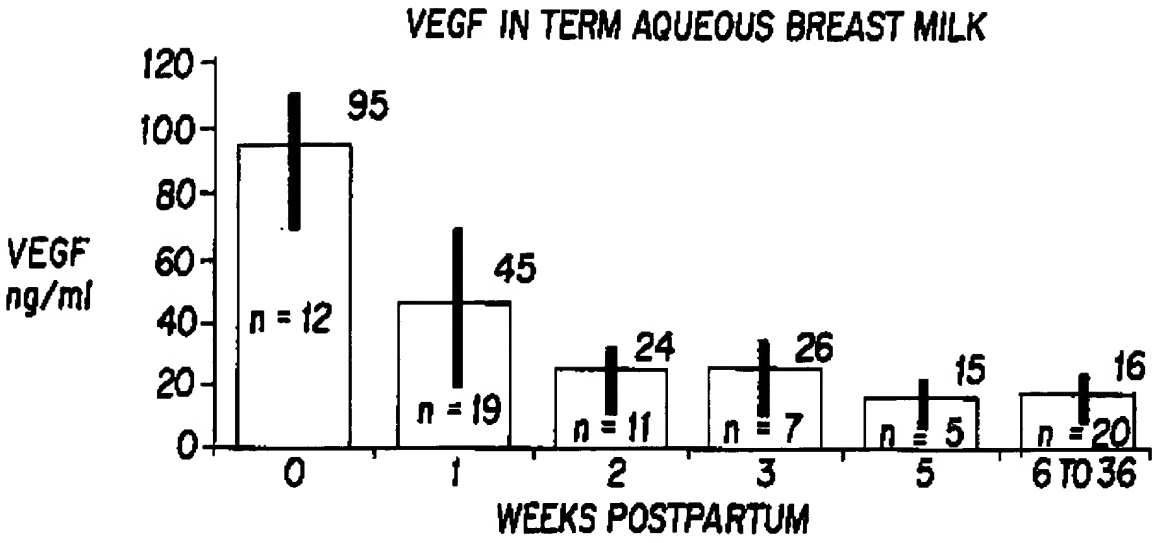
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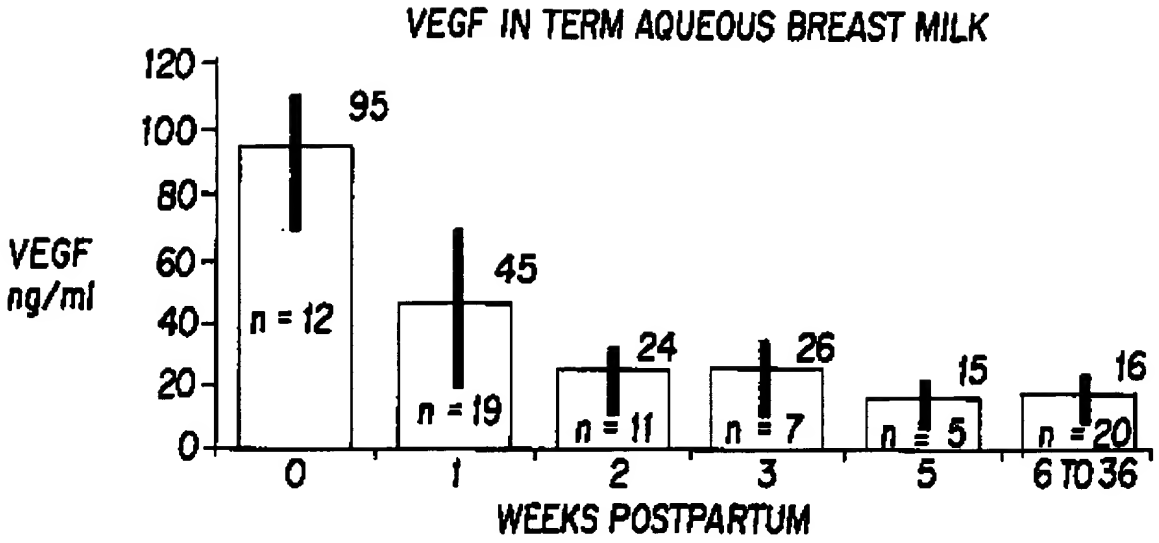
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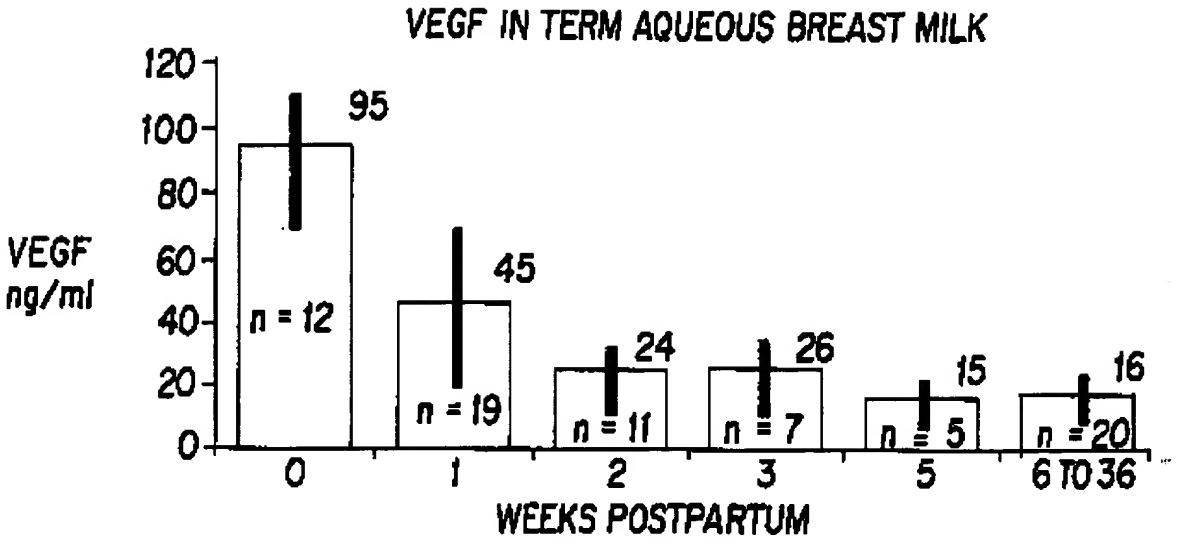
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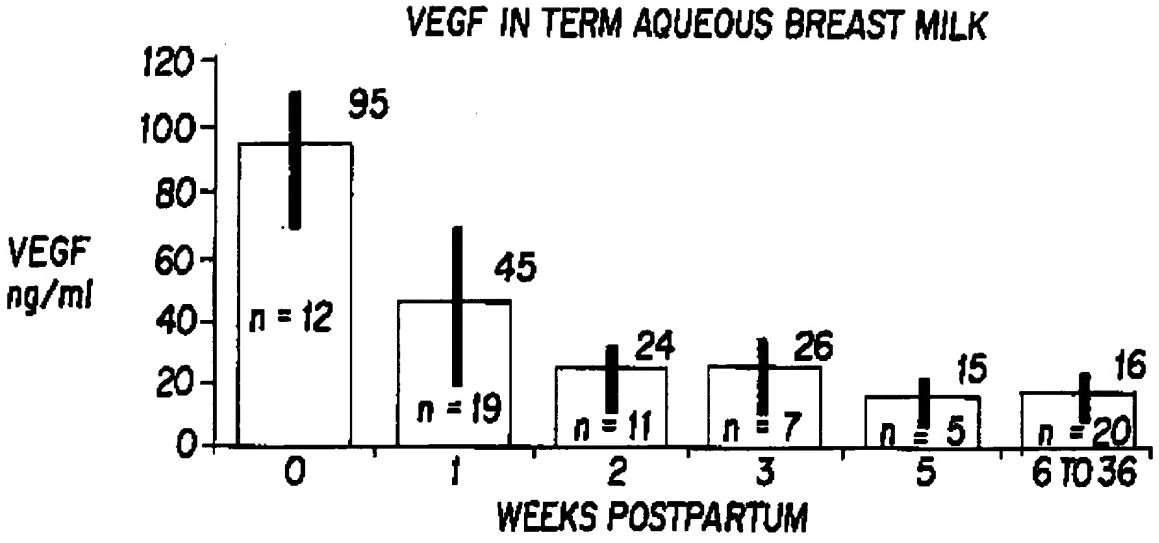
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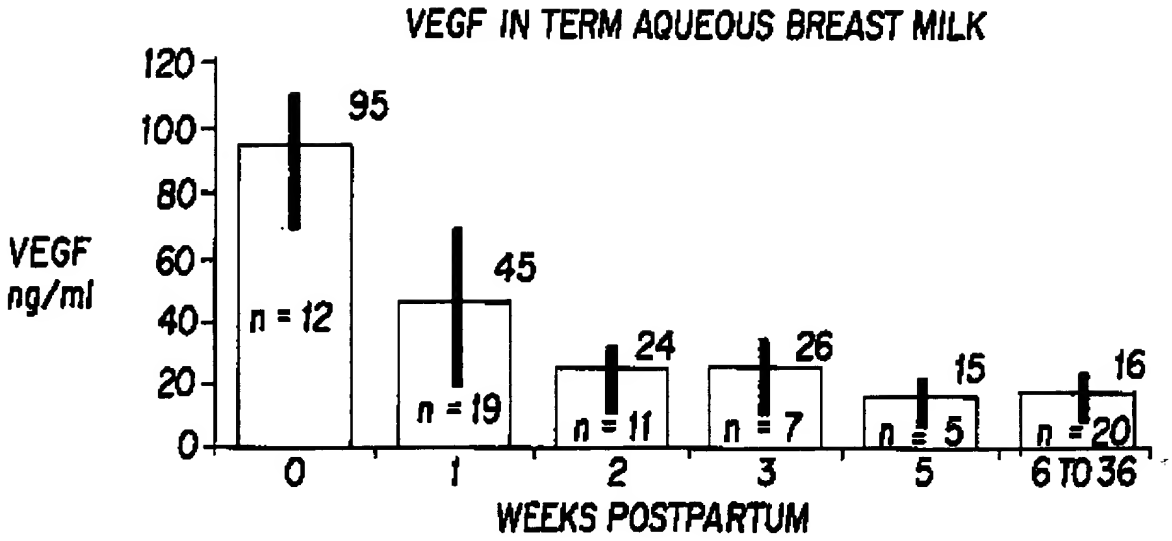
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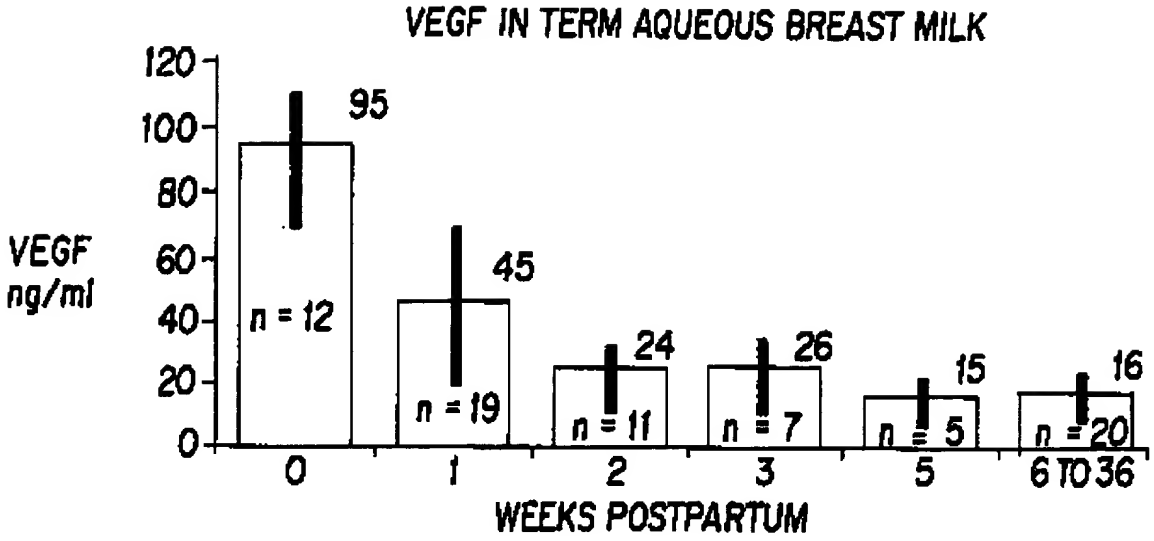
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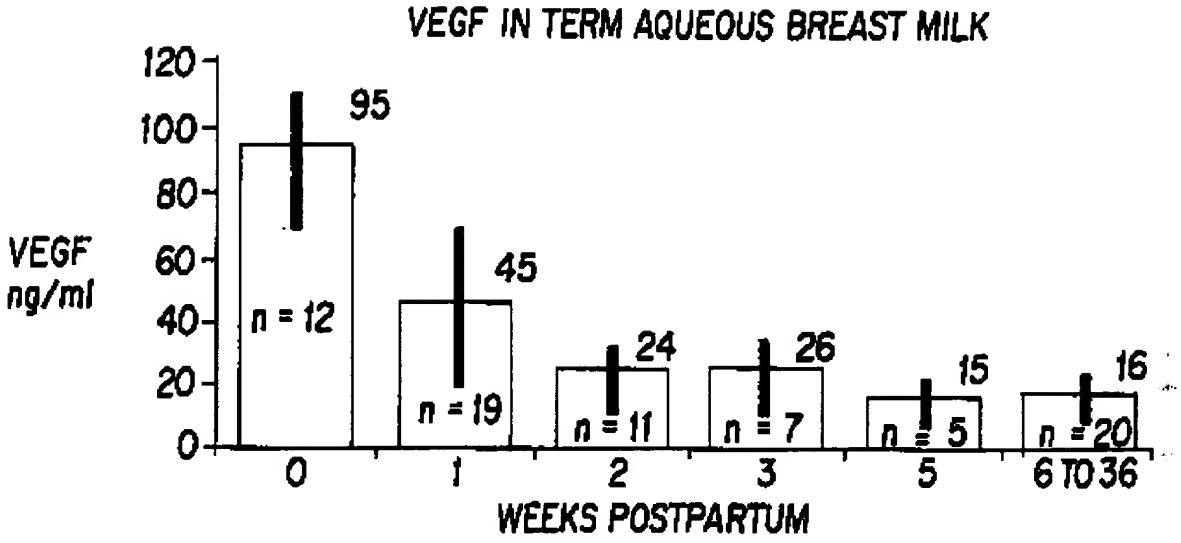
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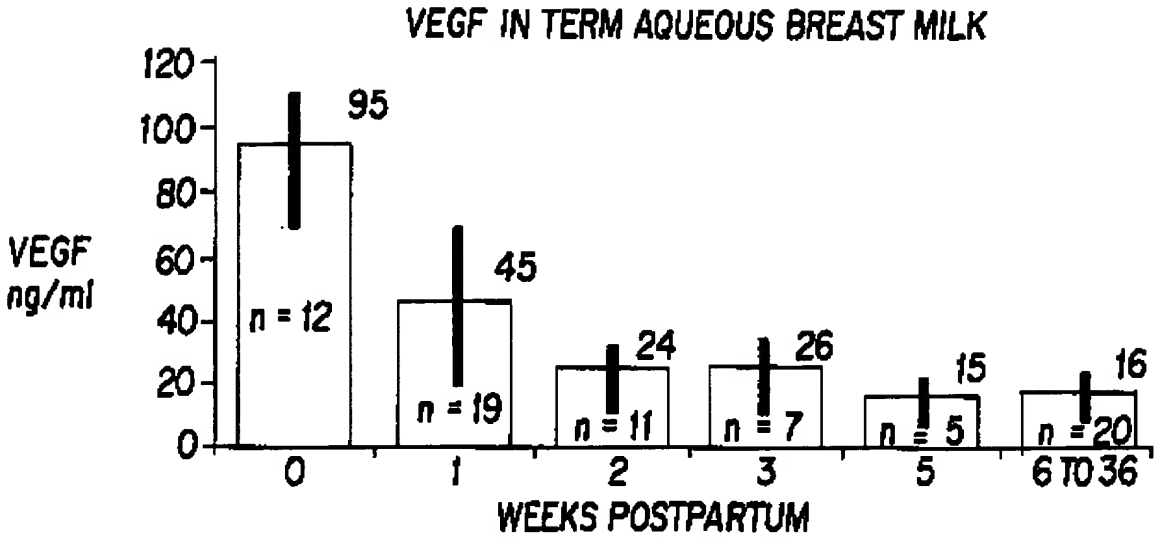
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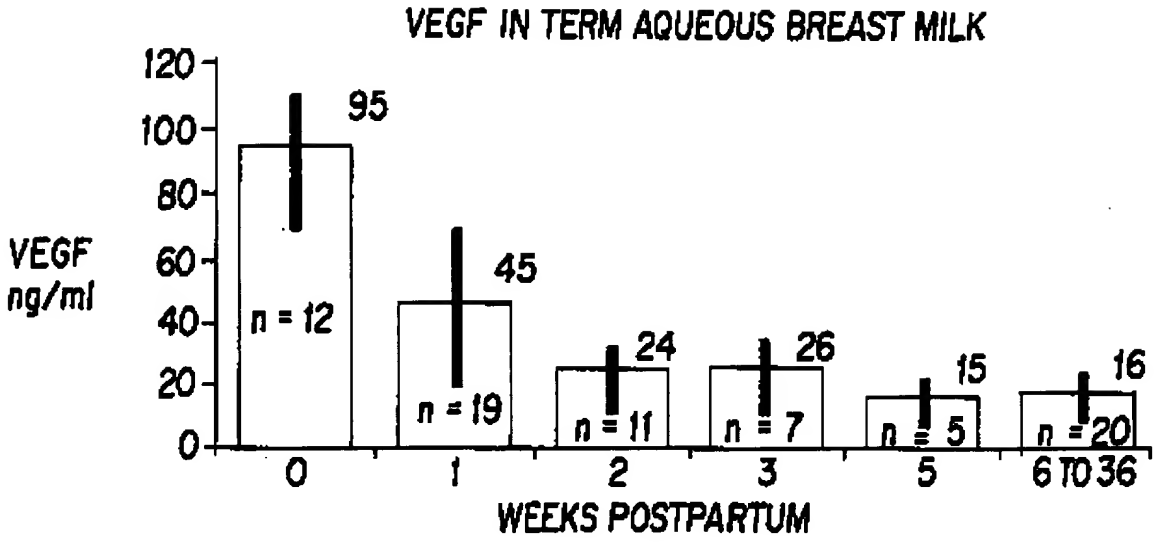
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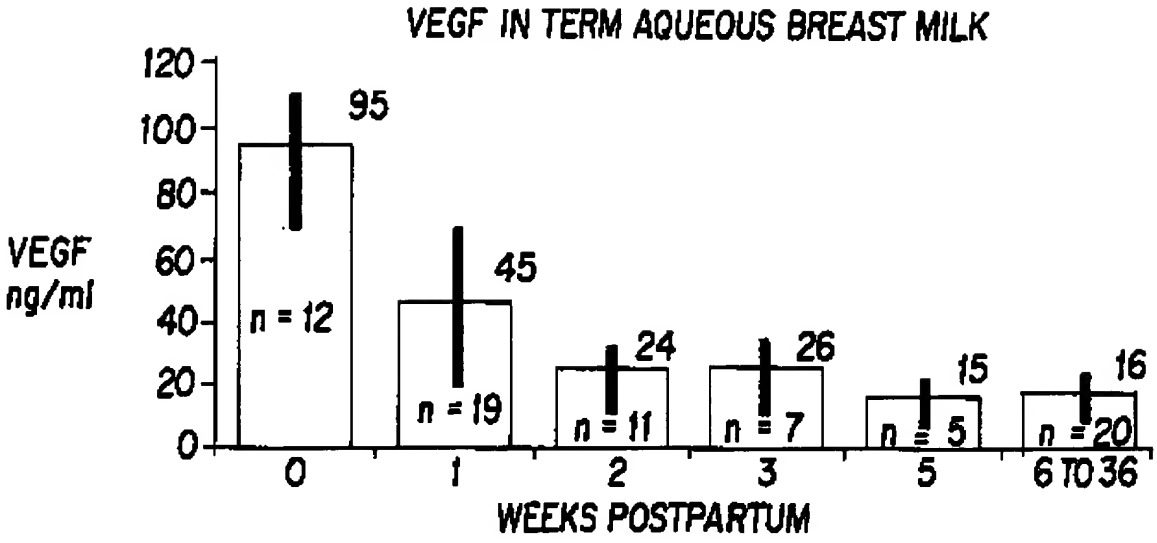
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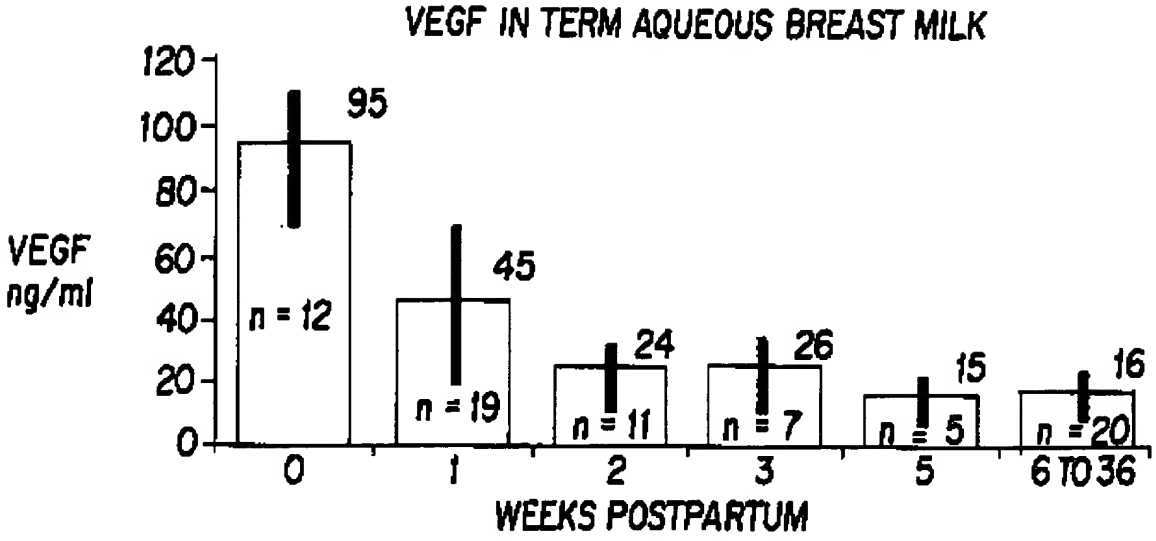
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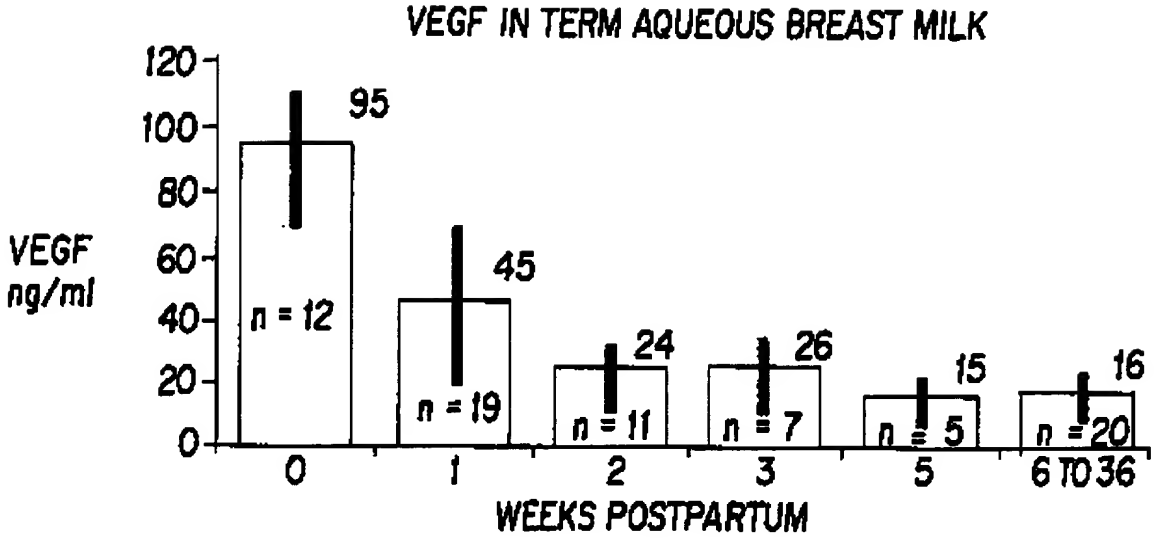
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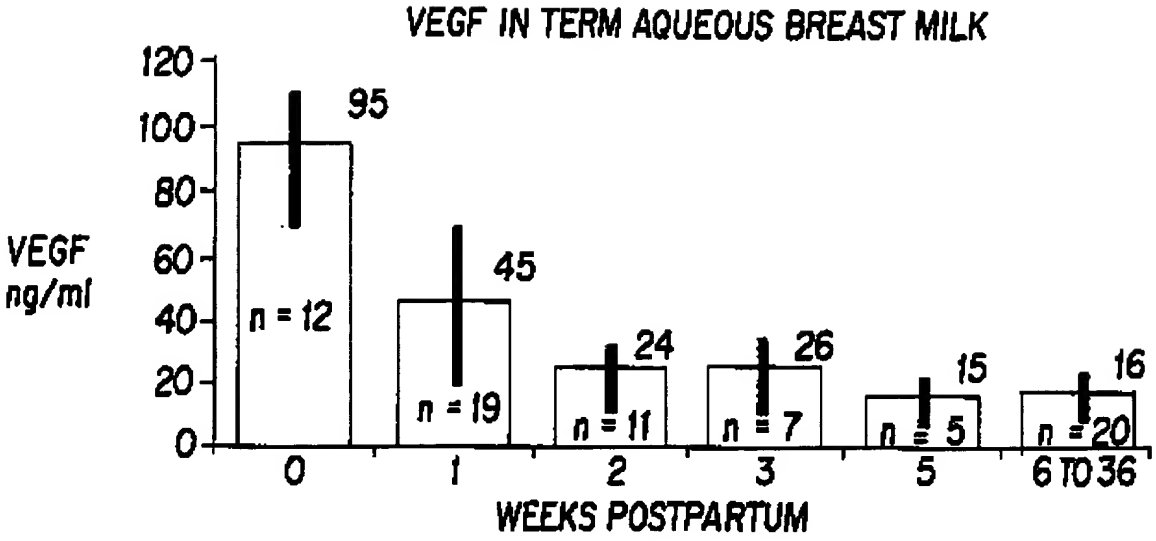
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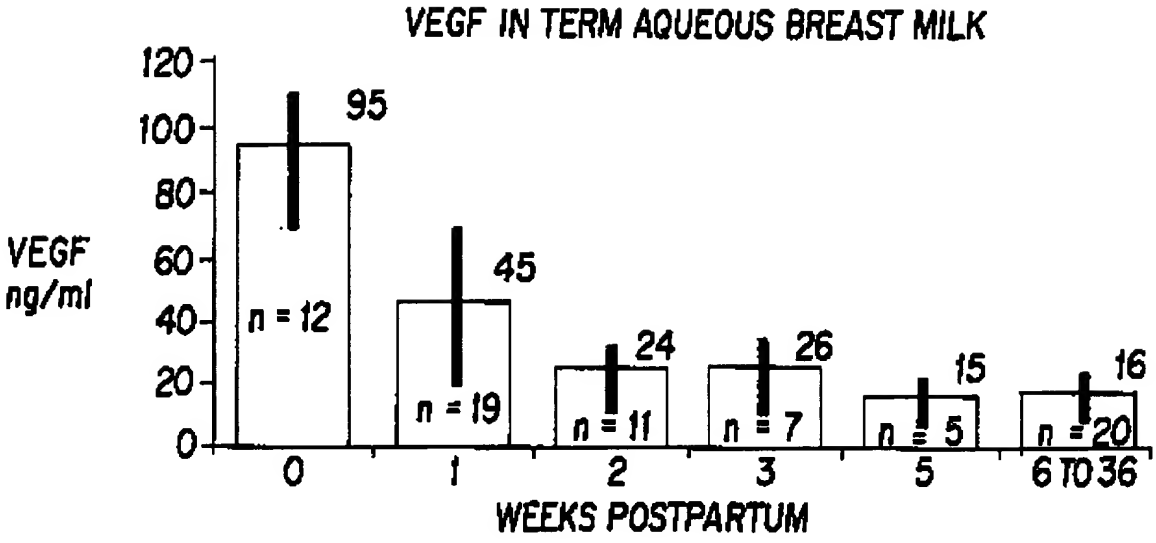
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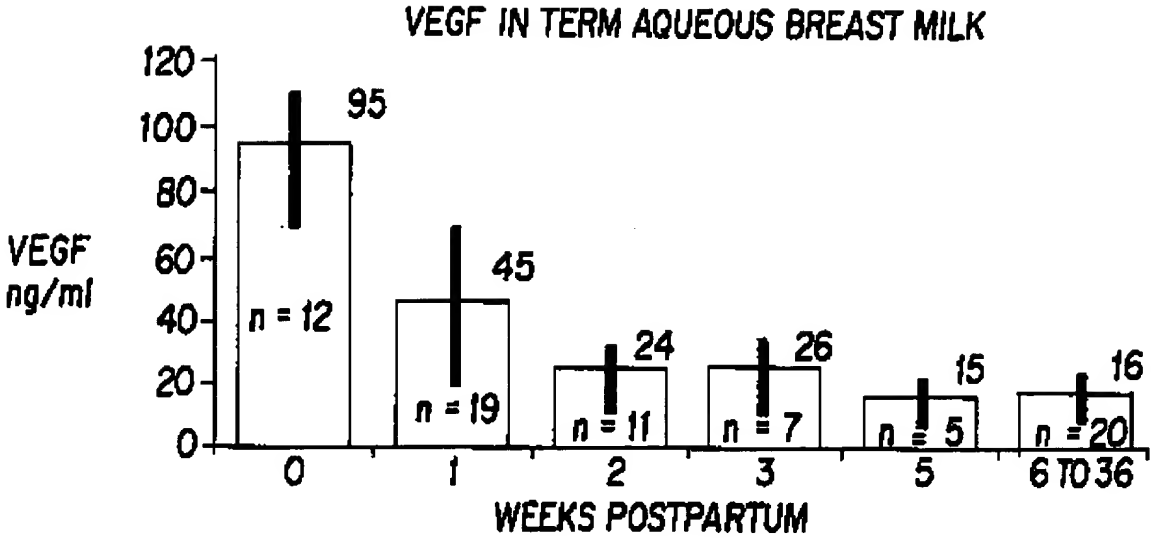
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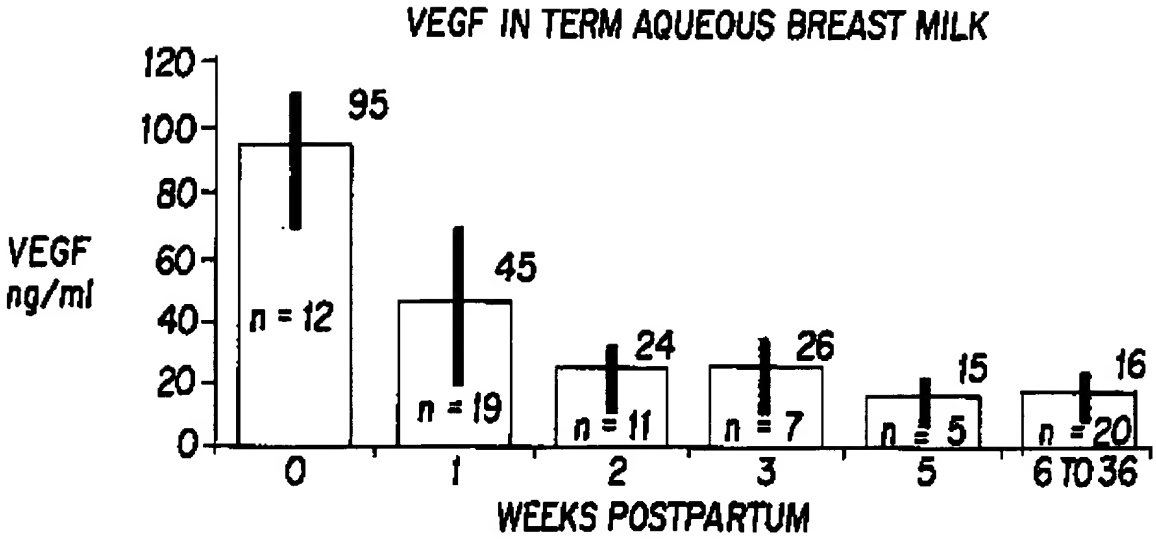
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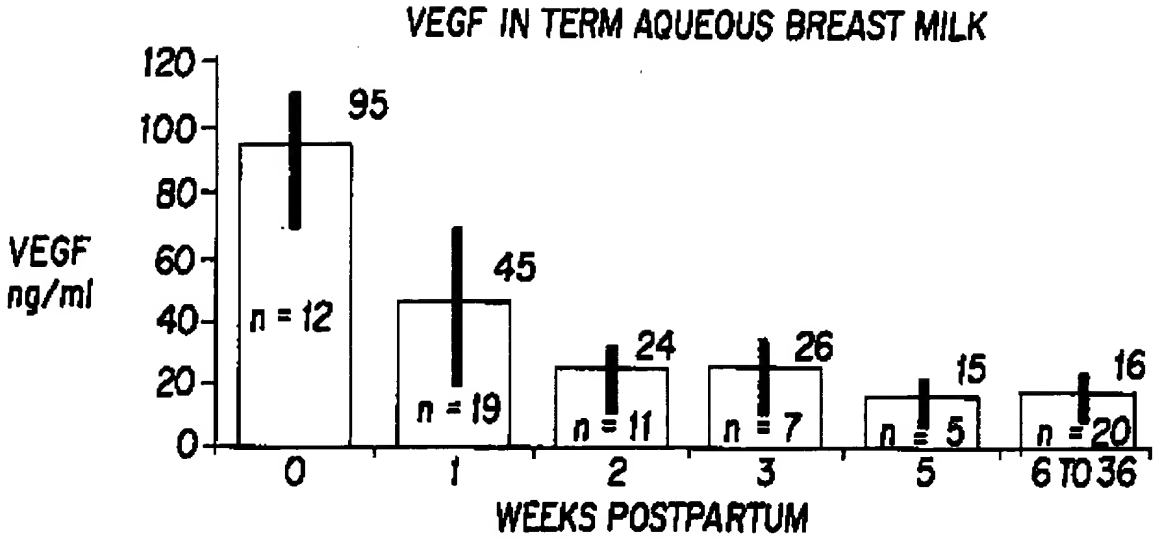
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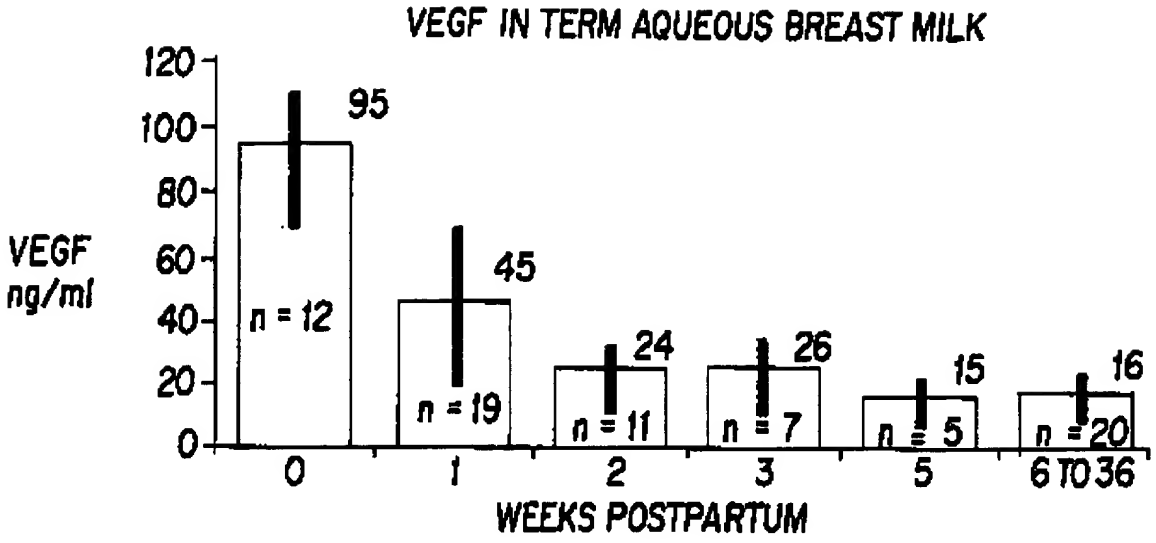
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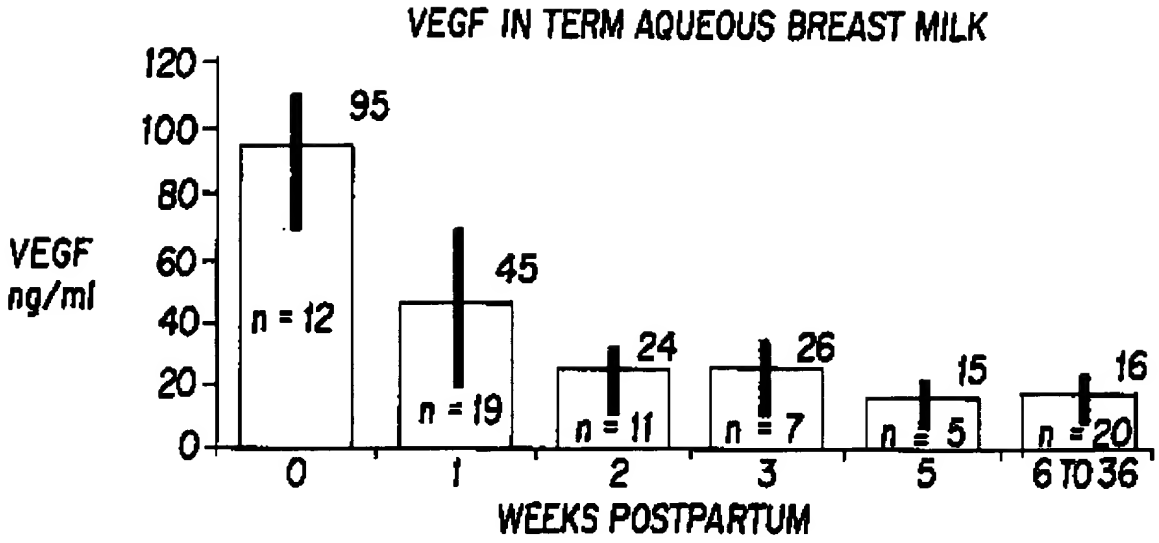
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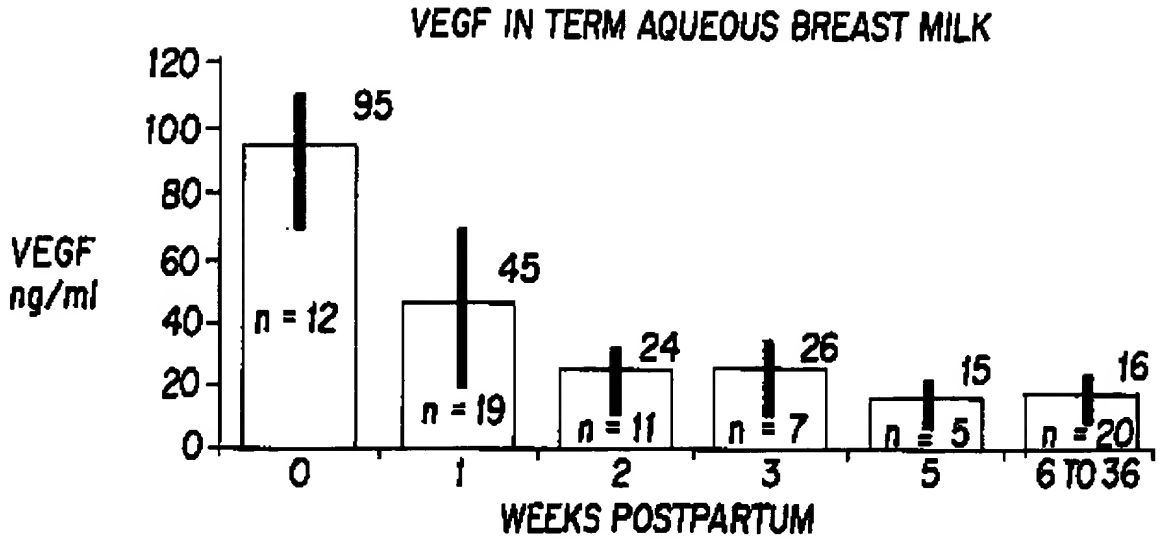
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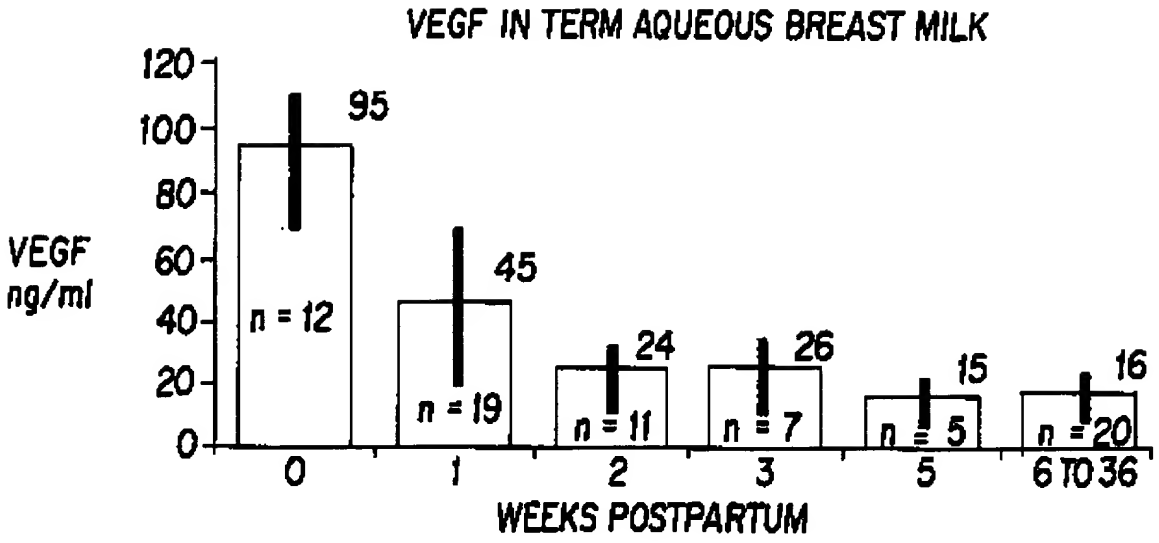
WEEKS POSTPARTUM

FIG. 4


VEGF IN TERM AQUEOUS BREAST MILK
VEGF ng/ml
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6 TO 36
WEEKS POSTPARTUM

FIG. 4

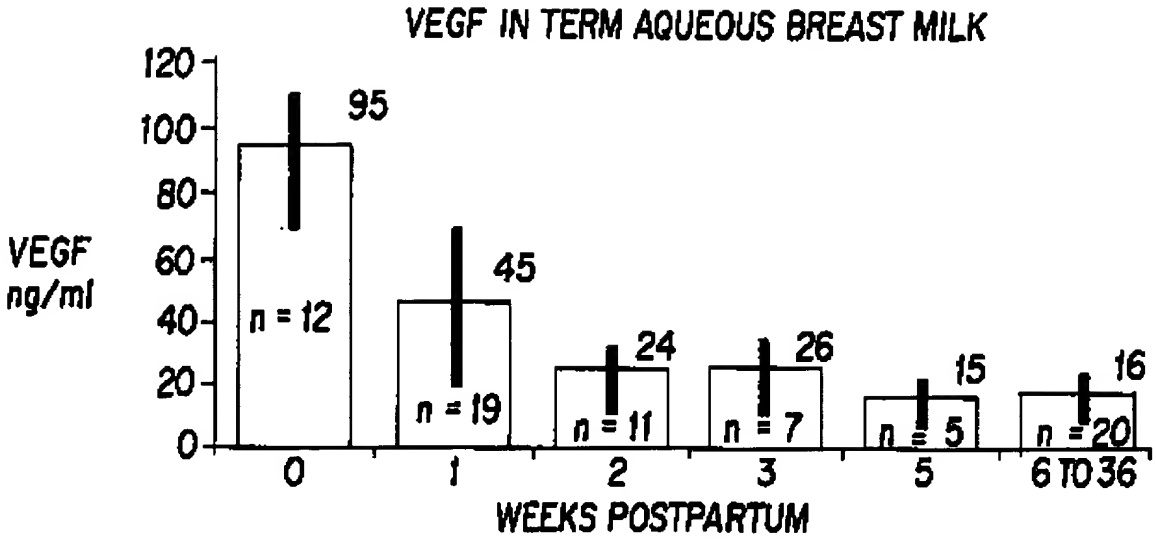
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FIG. 4

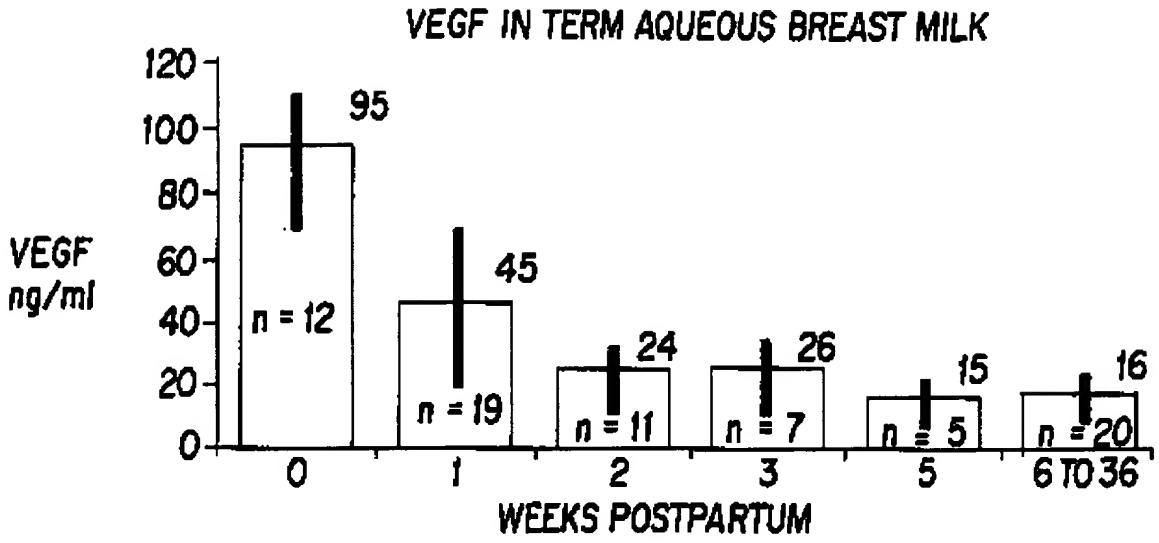
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FIG. 4

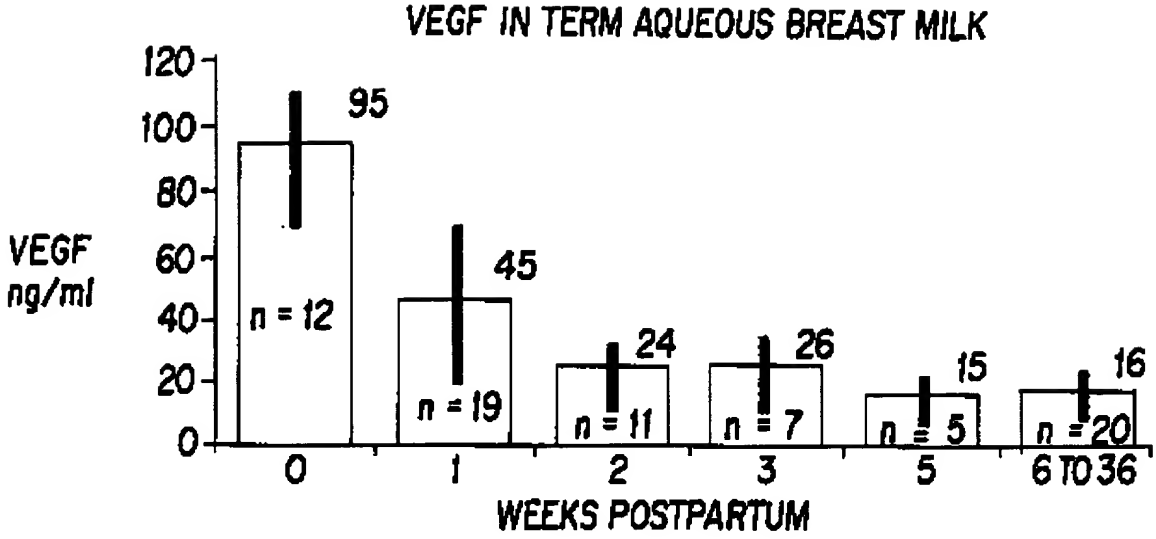
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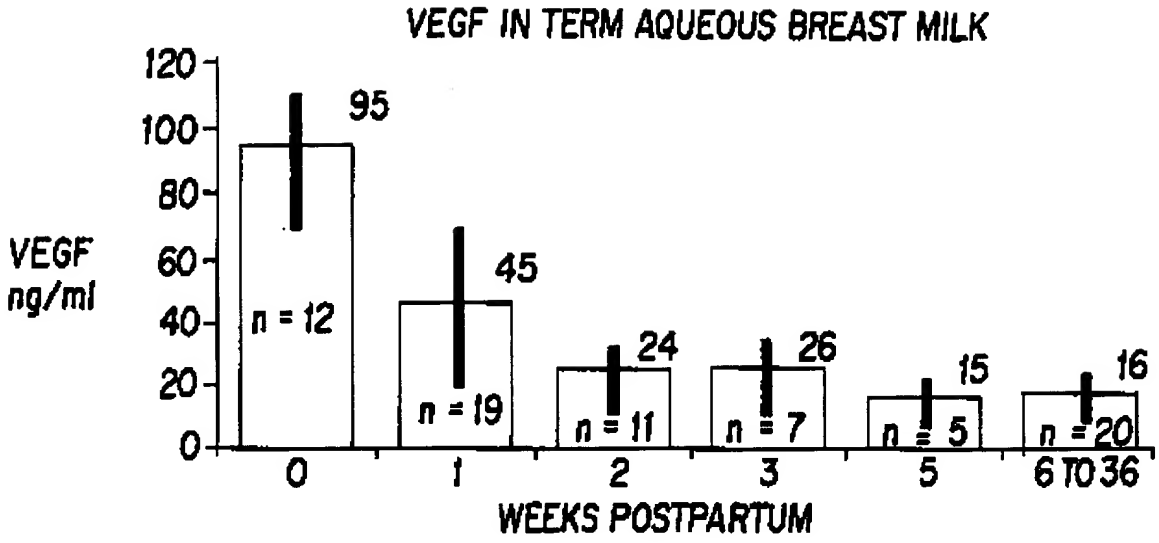
FIG. 4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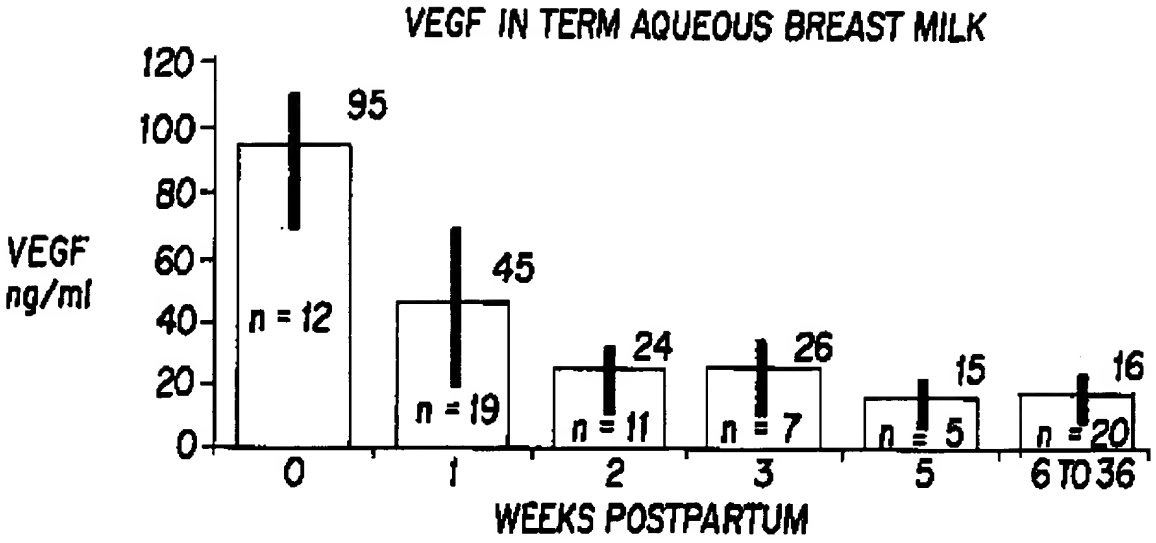
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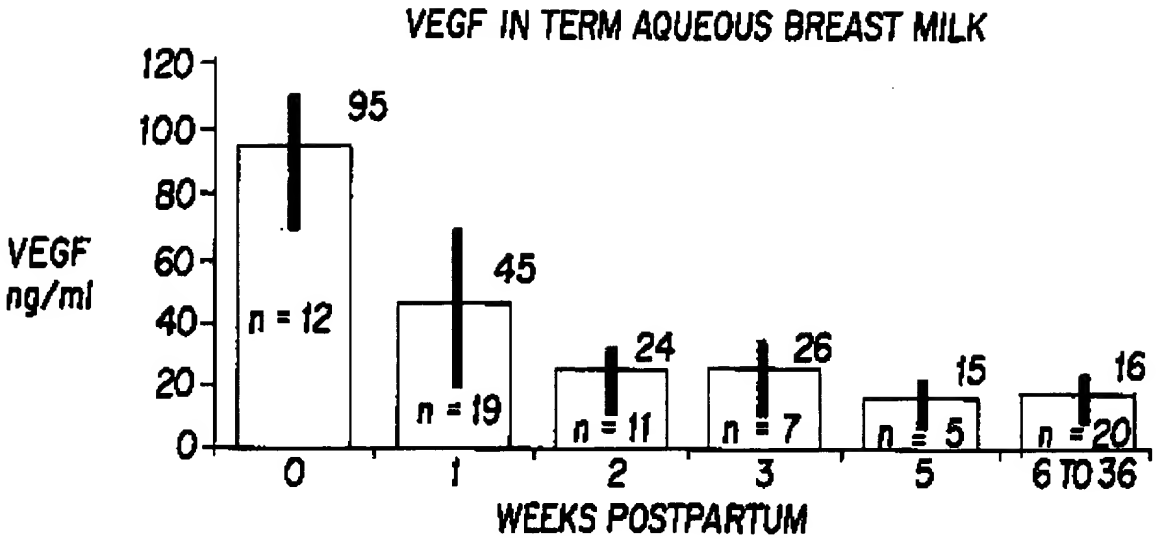
FIG. 4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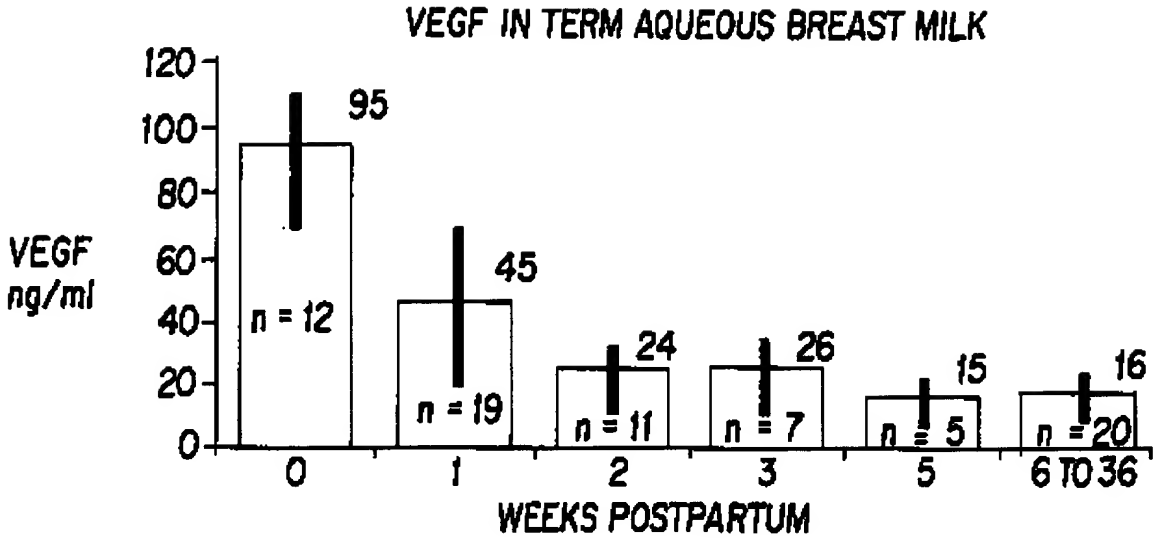
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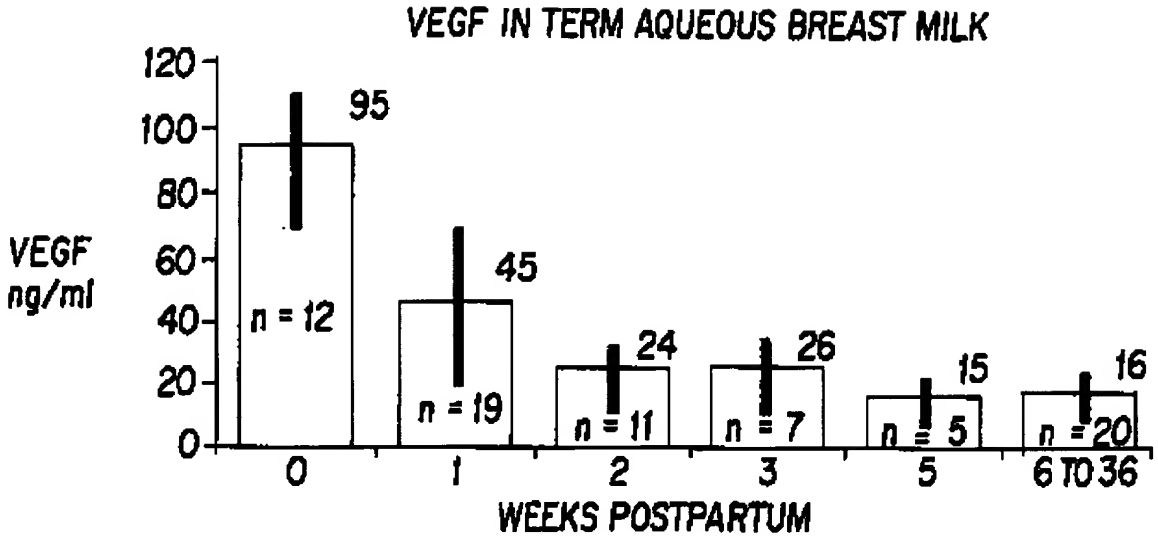
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VEGF ng/ml
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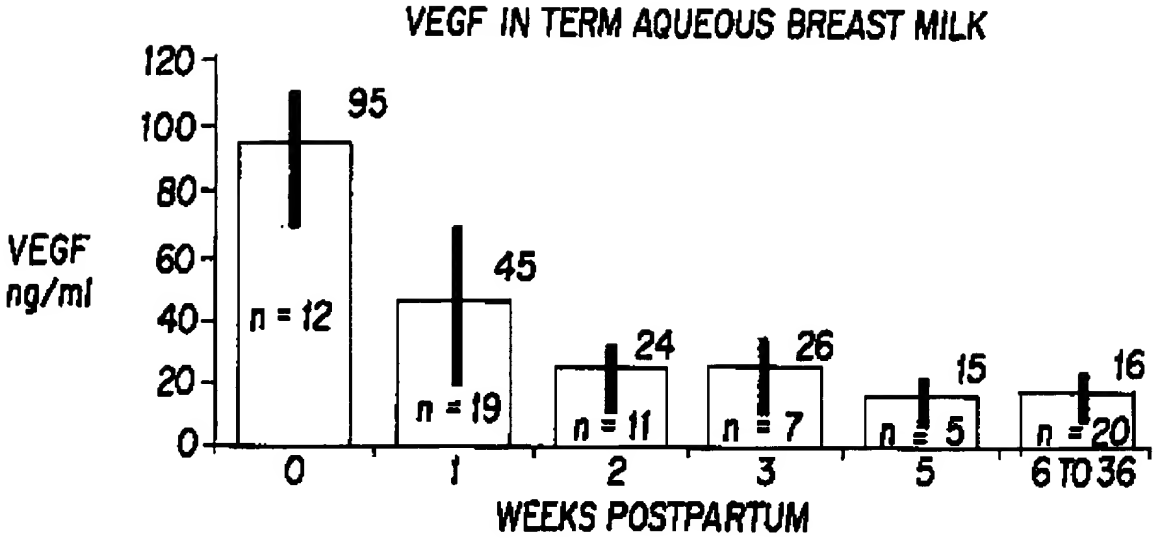
FIG. 4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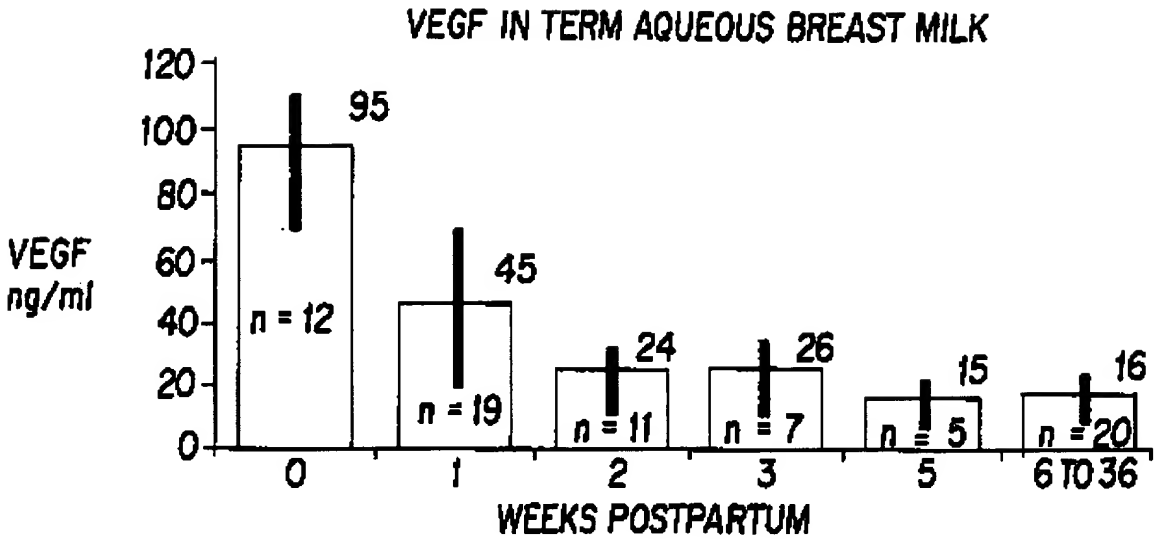
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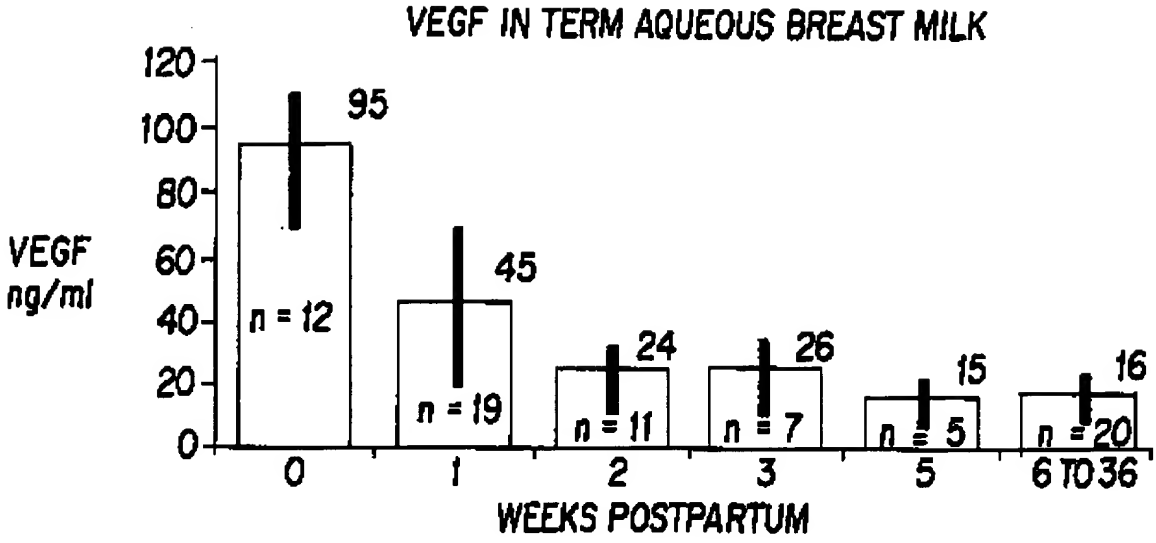
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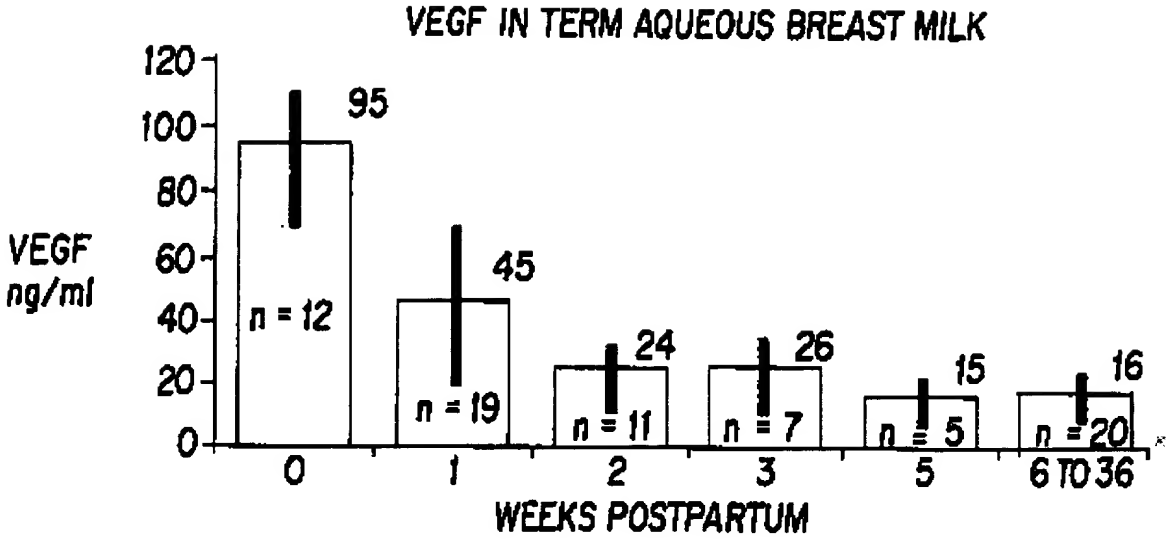
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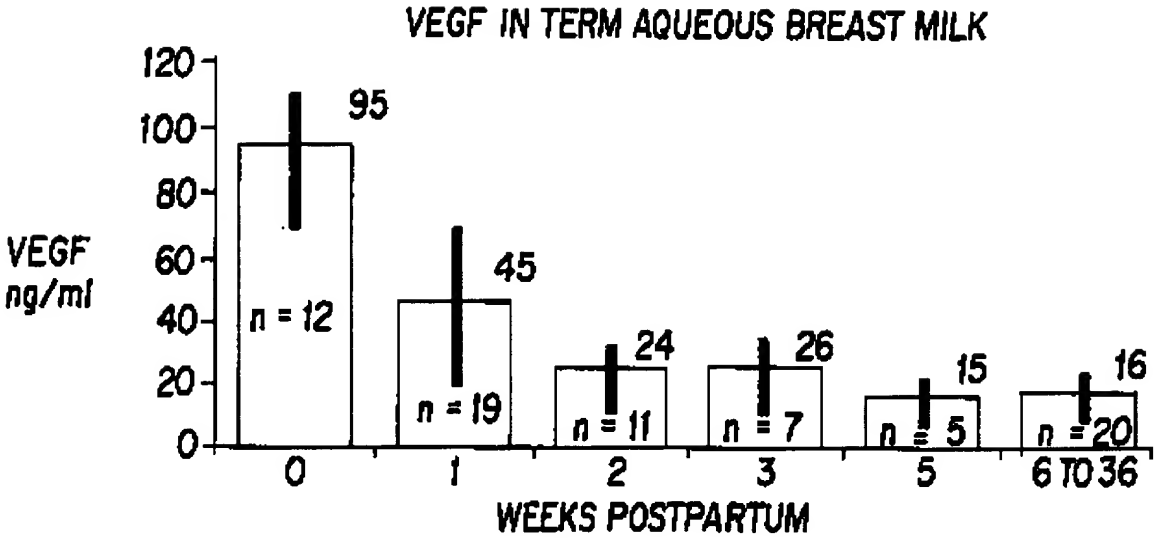
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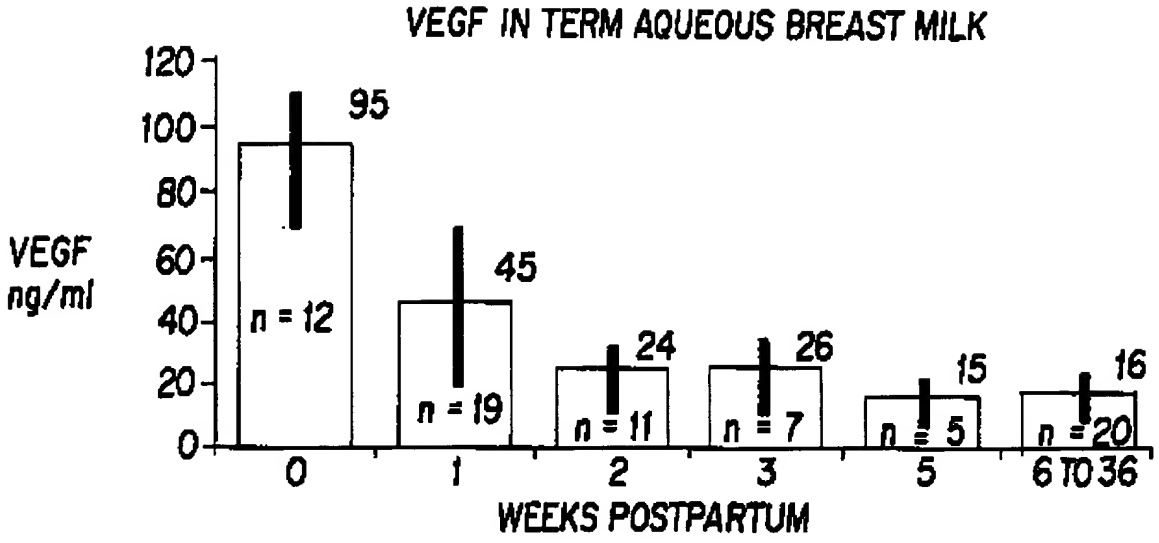
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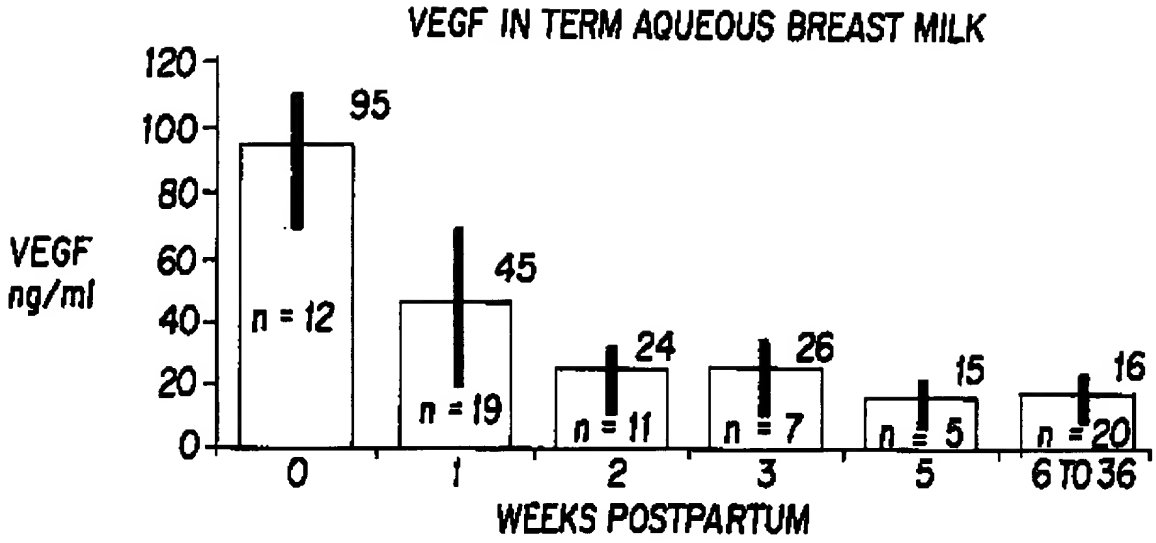
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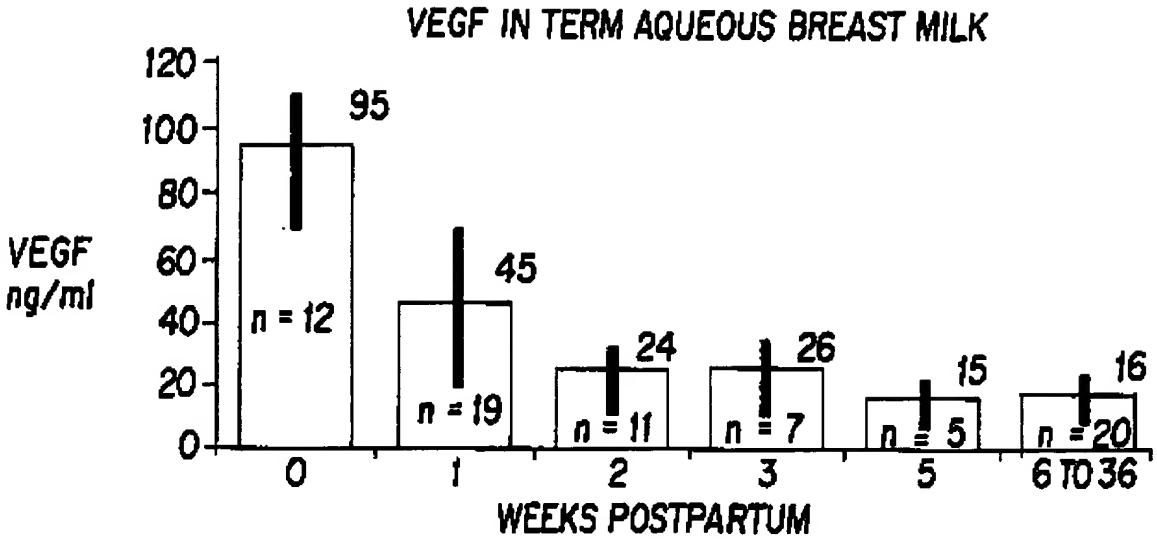
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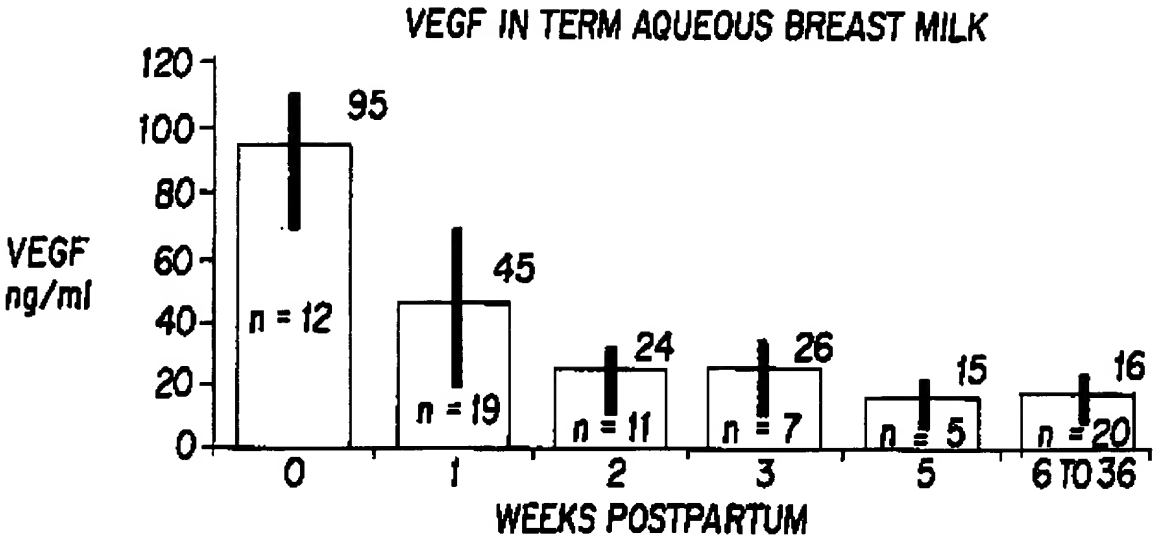
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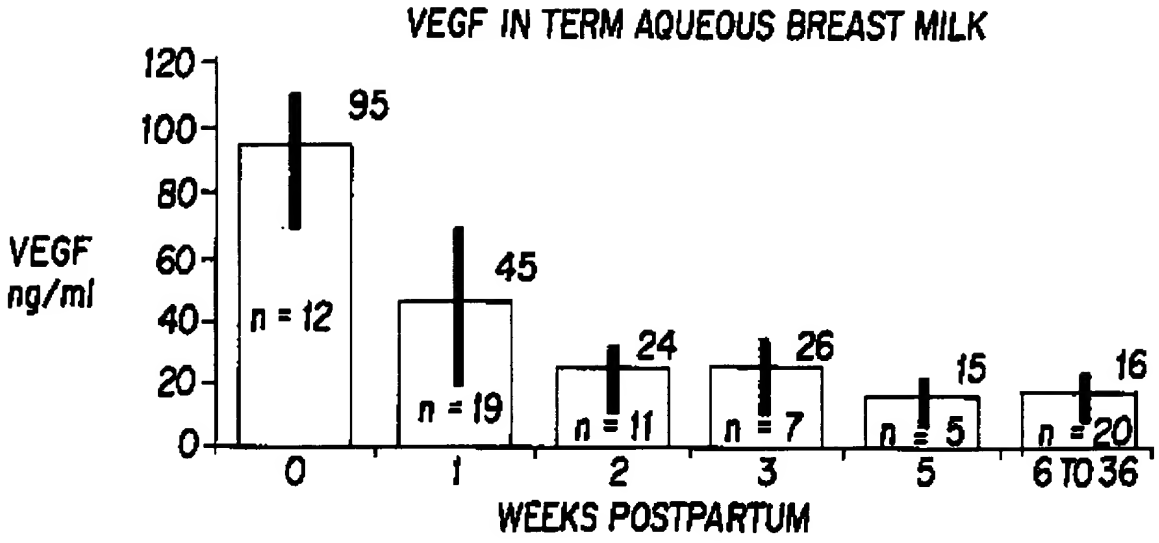
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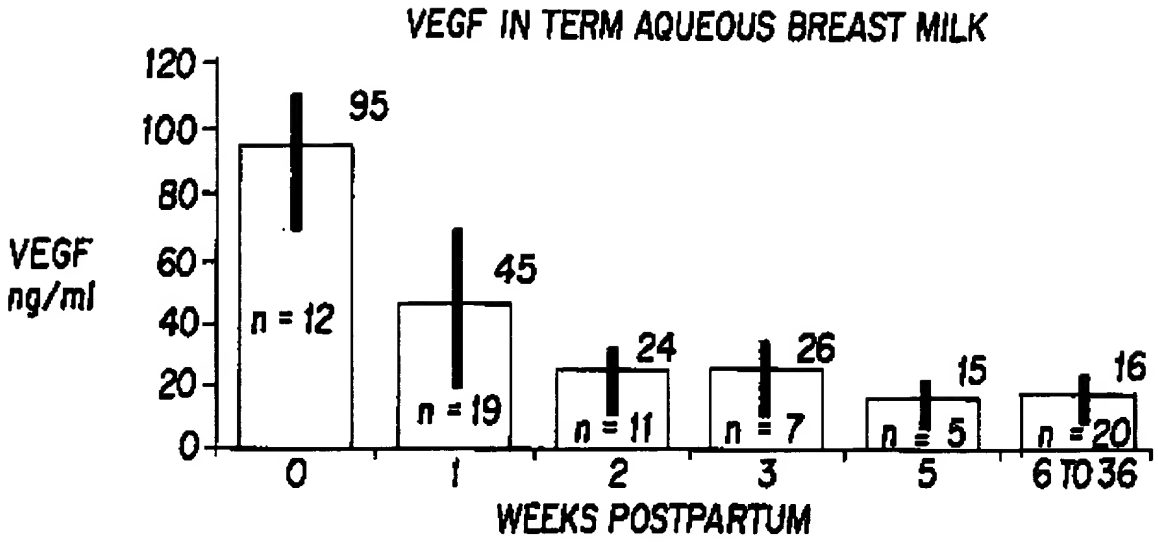
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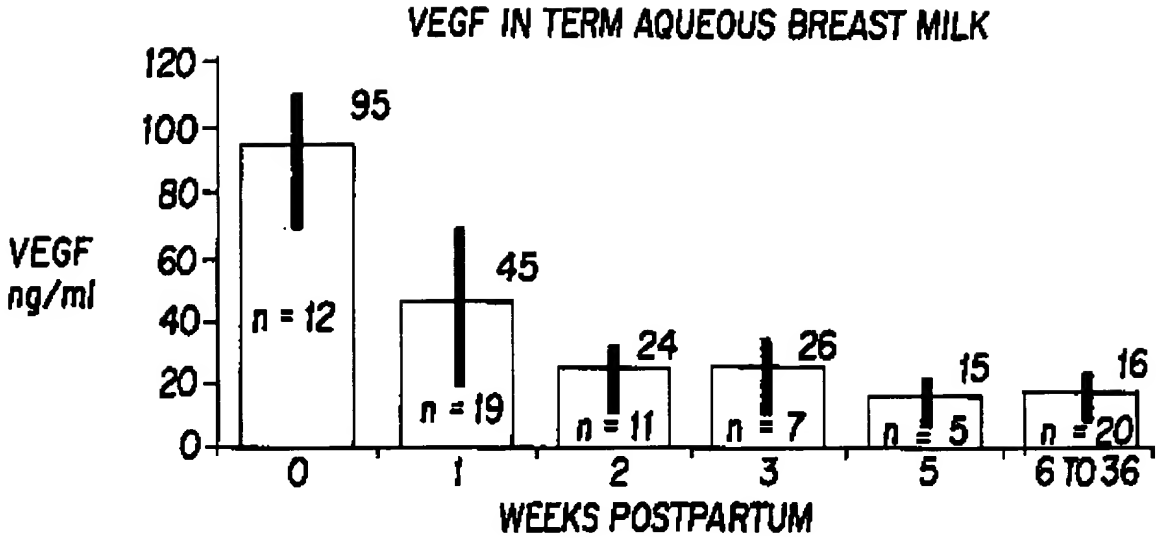
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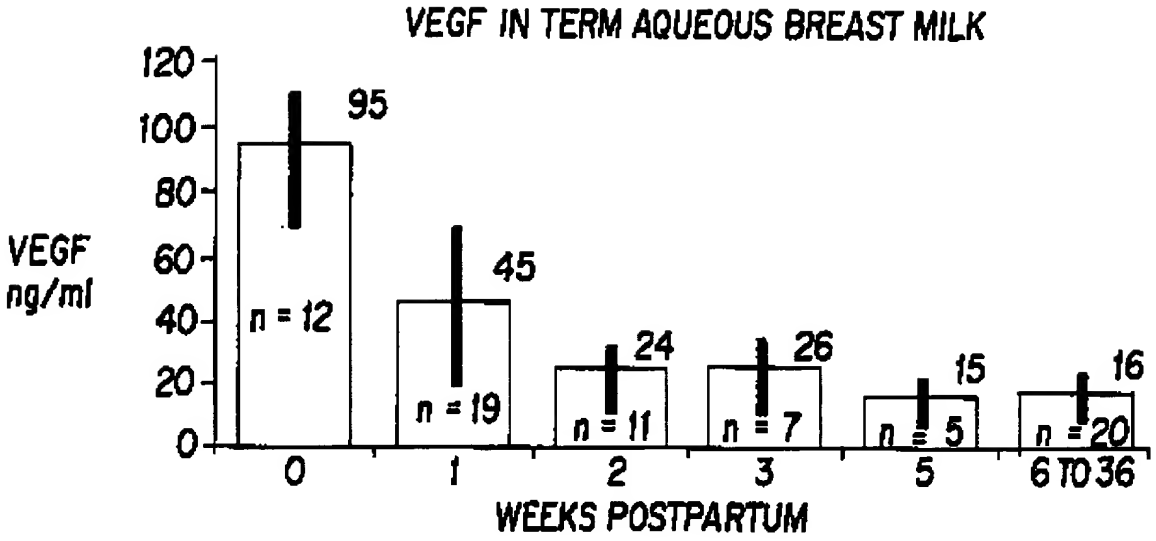
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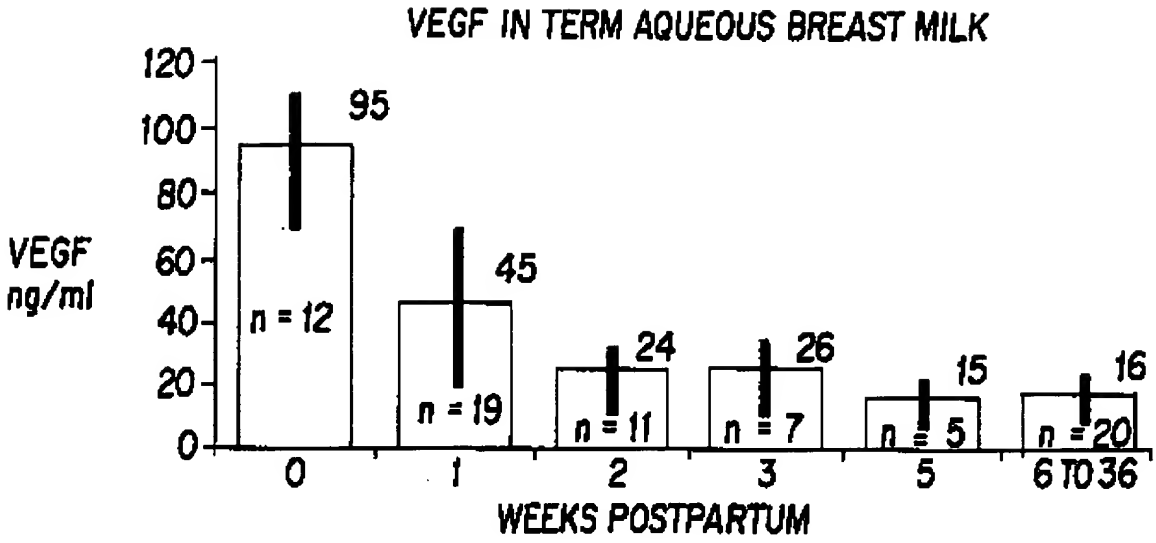
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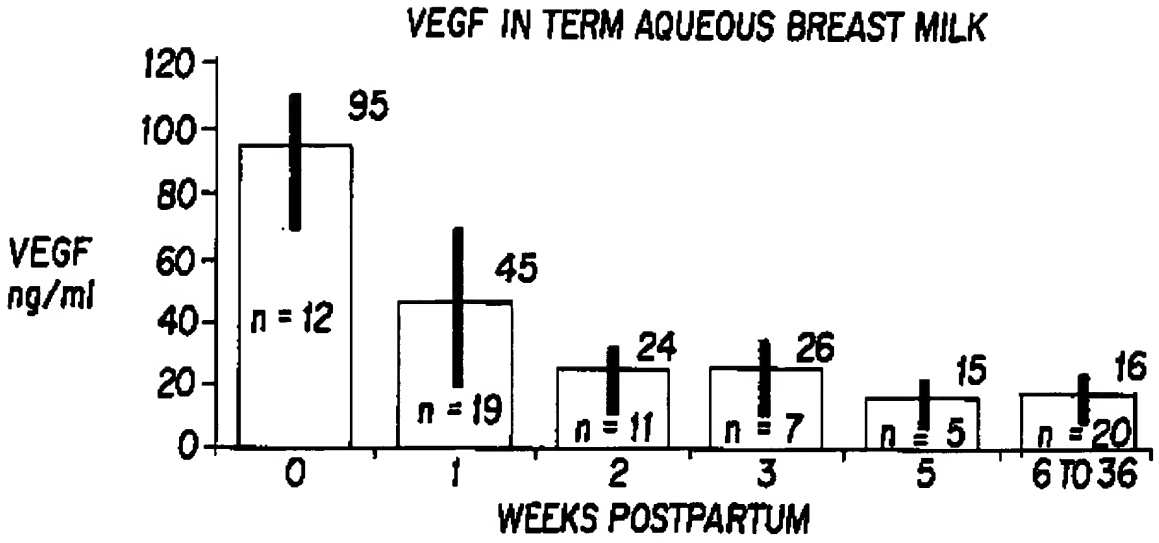
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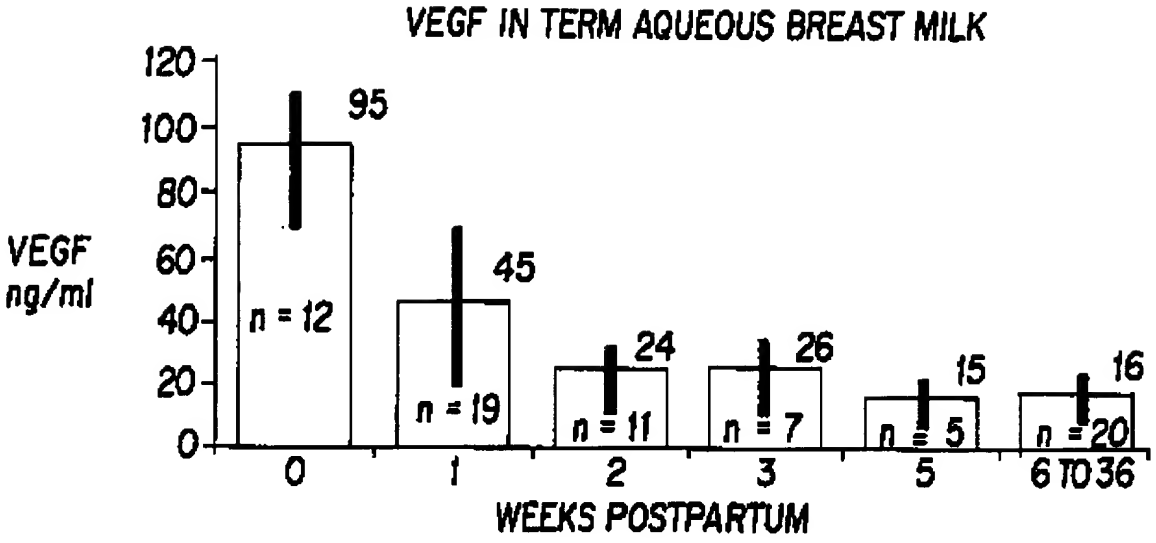
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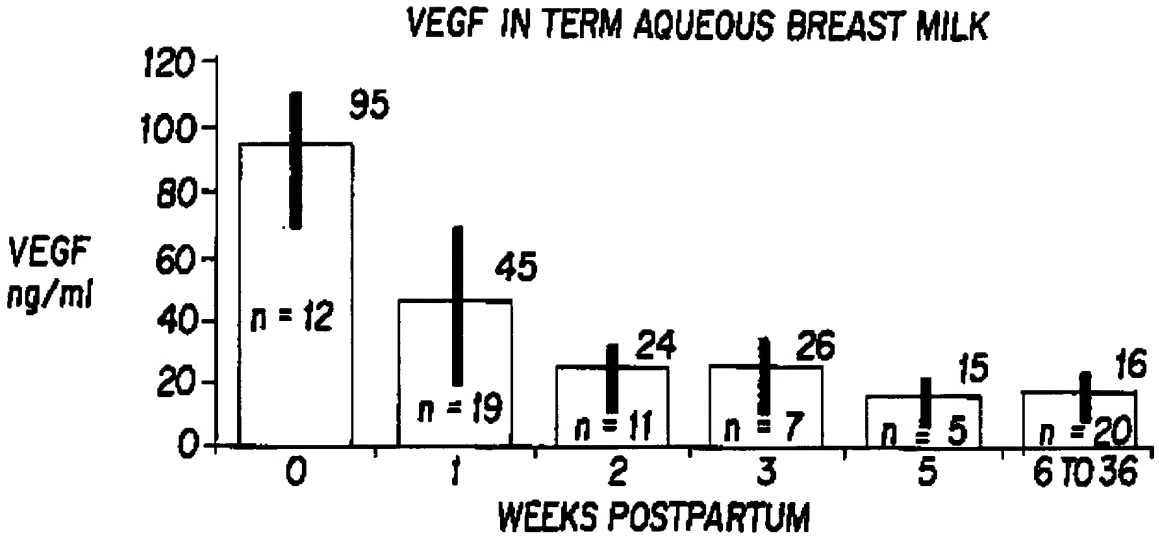
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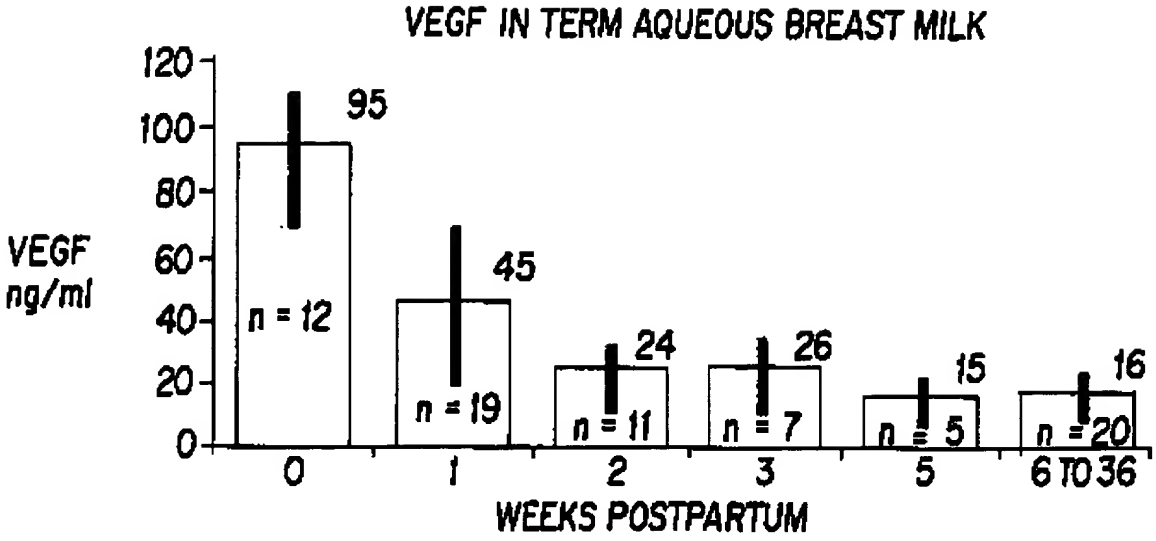
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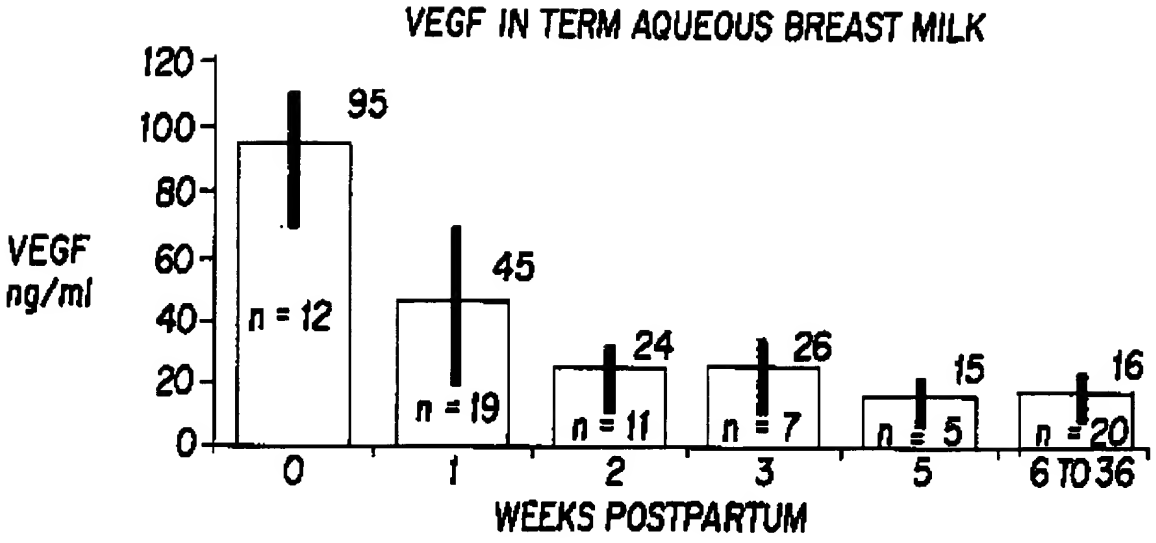
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